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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, C07H 15/12
C12N 15/00

A1

(11) International Publication Number: WO 91/11535

(43) International Publication Date: 8 August 1991 (08.08.91)

(21) International Application Number: PCT/US91/00635

(22) International Filing Date: 30 January 1991 (30.01.91)

(30) Priority data:
472,137 30 January 1990 (30.01.90) US
Not furnished 29 January 1991 (29.01.91) US

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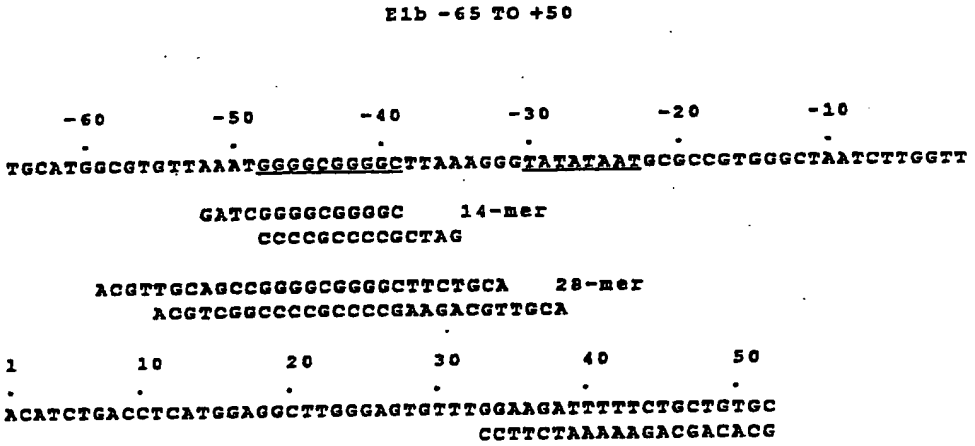
(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.

(54) Title: INHIBITION OF TRANSCRIPTION BY DOUBLE-STRANDED OLIGONUCLEOTIDES



(57) Abstract

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to inhibit the interaction of transcriptional factors with transcriptional control elements in DNA. The methods of the invention are particularly useful in selectively inhibiting transcription of viral genes and oncogenes, and may be used in the treatment of a variety of viral diseases. In preferred embodiments of the invention, nucleosides are joined by phosphorothioate linkage.

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INHIBITION OF TRANSCRIPTION BY DOUBLE-STRANDED OLIGONUCLEOTIDES

1. INTRODUCTION

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to inhibit the interaction of transcriptional factors with transcriptional control elements in DNA. The methods of the invention are particularly useful in selectively inhibiting transcription of viral genes and oncogenes, and may be used in the treatment of a variety of viral diseases. In preferred embodiments of the invention, nucleosides are joined by phosphorothioate linkage.

2. BACKGROUND OF THE INVENTION

The rate of initiation of transcription is regulated by cis-acting promoter and enhancer elements. The mechanism for this control involves binding of DNA sequence-specific proteins to these elements (Gidoni et al., 1984, Nature, 312:409-413; Scholer, H. R. and Gruss, P., 1984, Cell, 86:403-411; and McKnight, S. L. and Kingsburg, R., 1982, Science, 217:316-324). Regulatory elements and binding proteins have been identified by in vivo functional studies with plasmids or viruses containing mutated regions or competing binding sites. Alternatively, in vitro binding assays, such as footprinting and gel retardation analysis, have been useful in delineating promoter function (Wu, C., 1986, Nature, 1985, 317:84-87 and Singh et al., 1986, Nature, 319:154-158). These studies have characterized both ubiquitous factors that bind to diverse regulatory elements and are present in nuclear extracts from many different cell types and unique factors that bind to few promoters or enhancers and are

present in only a specialized cell type or stage of differentiation (Rosales et al., 1987, EMBO J., 6:3015-3025; Peterson et al., 1988, Mol. Cell. Biol. 6:4168-4178).

2.1. TRANSCRIPTION FACTORS AND THEIR ROLES IN GENE EXPRESSION

A wide variety of transcription factors have been described. Transcription factors (TFs) have been identified which are the products of oncogenes, such as the fos protein (Lucibello et al., 1988, Oncogene 3:43-52) and v-jun protein (Bos et al., 1988, Cell 52:705-712). A TF has been found to be associated with the epidermal growth factor (EGF) receptor gene (Kageyama et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:5016-5020). Other TFs have been observed to function in a tissue specific manner, such as lymphoid-specific TFs (Muller et al., 1988, Nature 336:544-551; Scheidereit et al., 1988, Nature 336:551-557), the liver specific TF LF-B1 (Frain et al., 1989, Cell 59:145-157) and pituitary-specific TFs (Bodner et al., 1988, Cell 55:505-518; Ingraham et al., 1988, Cell 55:519-529). A convulsant-induced increase of TF-encoding mRNA has been observed in rat brain (Saffen et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7795-7799) and Morgan et al. (1987, Science 237:192-197) reported that c-fos mRNA was induced in rat brain by seizures.

A number of viral TF or cellular TFs which interact with viral promoter/enhancer elements have been identified. These include TF derived from SV40, which appears to activate the SV40 late promoter in vitro (Beard and Bruggmann, 1988, J. Virol. 62:4296-4302), and nuclear factor EF-C, which occurs in human HepG2 liver cells and in other nonliver cell lines and which is observed to bind to the hepatitus B virus and polyoma virus transcriptional enhancer regions in vitro (Ostapchuk et al., 1989, Mol. Cell. Biol. 9:2787-2797). Another interesting TF is the

papillomavirus E2 transactivator protein. The E2 open reading frame of bovine papillomavirus type 1 encodes at least three TFs. These include positive as well as negative regulators of transcription (Lambert et al., 1987, cell 50:64-78; McBride et al., 1988 EMBO J. 7:533-540; Lambert et al., 1989, J. virol 63:3151-3154).

Human immunodeficiency virus type 1 (HIV-1), which causes acquired immunodeficiency syndrome (AIDS), has been found to utilize a number of different TFs in its mode of gene expression, which interact with both viral and cellular proteins. Five regions of the HIV-1 long terminal repeat (LTR) region, including the negative regulatory enhancer, SP1, TATA, and TAR regions, have been shown to be important in the transcriptional regulation of HIV genes (Garcia et al., 1989, EMBO J. 8:765-778; Harrich et al., 1989, J. Virol. 63:2585-2591). The enhancer element has been found to contain two copies of the sequence GGGACTTCC (which shares homology with the eukaryotic TF NF-kappa B); the TAR region, located between -17 and +44 in the viral genome, contains two copies of the sequence CTCTCTGG (Wu et al., 1988, EMBO J. 7:2117-2130). Cellular protein EBP-1 has been observed to bind to the enhancer region, whereas cellular protein UBP-1 appears to bind to the TAR region (Wu et al., ibid). Further, a protein encoded by the human T cell leukemia virus I (HTLV-I) tax gene has been found to interact with the HIV-1 enhancer region. The HIV-1 transcriptional transactivator protein tat has also been shown to interact with the TAR region (Arya et al., 1985, Science 229: 69-73; Fisher et al., 1986, Nature 320:367-371; Kao et al., 1987, Nature 330:489-493).

Three SP-1 binding sites in the HIV LTR were found to be important to in vitro transcription from the HIV-LTR promoter. Harrick et al. (1989, J. Virol. 63:2585-2591) observed that mutagenesis of the HIV-1 LTR

SP-1 sites, which converted them to consensus high affinity SP-1 binding sites, resulted in an increase of tat-induced gene expression.

SP-1 is a ubiquitous factor which increases transcription of RNA polymerase II 10 to 50 fold from promoters that contain one or more hexanucleotide sequences, GGGCGG, called GC boxes (Gidoni, supra and Briggs et al., 1986, Science, 234:47-52).

The E1B transcriptional unit promoter has only a single SP1 binding site and a TATA box (Wu et al., 1987, Nature, 326:512-515). Deletion and linker scan substitution of the SP1 site in the adenovirus E1B promoter produced mutant viruses that yielded only 13 to 20% of the basal transcription of wild type virus after infection of HeLa cells (Wu, supra).

2.2. OLIGONUCLEOTIDE INHIBITORS OF TRANSCRIPTION

Currently, investigators are attempting to control the expression of genes with single stranded antisense oligonucleotides. Antisense oligonucleotides bind to complementary mRNA sequences and block translation or cause cleavage of the double stranded duplexes formed (Stein, C. A. and Cohen, J. S., 1988, Cancer Res., 48:2659-2668). This approach can decrease expression of specific genes but cannot be generally applied because of limited binding of the oligonucleotide to mRNA regions of strong secondary structure, poor transport into cells and rapid degradation. More stable chemically modified oligonucleotide have multiple stereoisomers that may decrease binding to mRNA (Zon, 1988, supra).

Other investigators have explored the use of double-stranded oligonucleotides in controlling gene expression (European Patent Application No. 88307302.5; Androphy et al., 1987, Nature 325:70-72), but have faced

the problem that such oligonucleotides do not pass readily into cells, and are susceptible to degradation. For example, Hawley-Nelson et al. (1988, EMBO 7:525-531) inserted oligonucleotides into a plasmid and then used standard calcium phosphate transfection techniques to introduce the oligonucleotides into eukaryotic cells. Such transfection techniques, to date, cannot be feasibly applied to introduce DNA into living organisms.

3. SUMMARY OF THE INVENTION

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to compete for the binding of nuclear factors to specific cellular transcriptional control elements. The invention is based in part on the discovery that oligonucleotides containing a GC box can specifically inhibit transcription of E1B.

In various embodiments of the invention, an oligonucleotide comprising one or, preferably, more than one binding site for a transcription factor may be used to inhibit the transcription of genes under the control of promoter/enhancer elements which bind to said transcription factor. In preferred embodiments of the invention, the transcription factor is a viral transcription factor, and the method of the invention may be used in the treatment of viral diseases, such as retroviral diseases, in humans or animals. In other preferred embodiments of the invention, the transcription factor binds to a control element of an oncogene or growth factor, and the method of the invention may be used in the treatment or prevention of cancer. According to the most preferred embodiments of the invention, the nucleosides of the oligonucleotide are joined by phosphorothioate linkage.

In an alternate embodiment of the invention, a oligonucleotide comprising one or more than one binding site for a transcription factor may be used to increase the transcription of genes normally repressed by said transcription factor.

According to a specific embodiment of the invention, oligonucleotides containing a SP1 binding sequence may be used to inhibit transcription of E1B. In a preferred specific embodiment of the invention, oligonucleotides may comprises multiple copies of SP1 binding sequences.

4. DESCRIPTION OF THE FIGURES

Figure 1. Nucleotide sequence of the E1B transcriptional unit and the inhibitor oligonucleotides.

The first line is the promoter region of E1B with the GC and TATA boxes underlined. The sequences of the annealed 14 mers and 28 mers are shown on the next 4 lines oriented below the homologous GC box in the E1B promoter. The transcribed portion of E1B (+1 to +52) is shown on the next line with the synthetic oligonucleotide used for the primer extension oriented below.

Figure 2. Radioautograph of a transcription assay. The heavy bands at the bottom are the 20 base oligomer used for the primer extension. The 50 base bands are the expected transcript for the E1B unit shown in Fig. 1. Reactions in lane 1 and 2 contained no competing oligonucleotides. Lanes 3 and 4 contained 0.22 and 0.87 μg 14 mer, SP1S. Lanes 5 and 6 contained 0.2 and 1.0 μg of the 28 mer, 17/19; lanes 7, 8 and 9 contained 0.12, 0.8 and 1.0 μg of 21/25; and lanes 10, 11 and 12 contained 0.1, 0.8 and 1.0 μg of 18/20. M is a standard lane of molecular weight markers containing labeled HpaII digest of pBR322.

Figure 3. Inhibition of transcription by varying concentrations of oligonucleotides with one, two or three SP1 sites. The lines are best fit regressions through the data.

Figure 4. Uptake of radiolabelled phosphodiester-linked (open circles) and phosphorothioate linked (closed circles) oligonucleotides by MOLT 4 cells.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel methods for controlling gene expression in which double stranded oligonucleotides are used to competitively inhibit the binding of transcription factors to specific transcriptional control elements in DNA. For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) transcription factors which may be inhibited according to the invention;

(ii) identification of oligonucleotides that may be used to inhibit transcriptional factor binding to control elements;

(iii) oligonucleotides of the invention; and

(iv) utility of the invention.

5.1. TRANSCRIPTION FACTORS WHICH MAY BE INHIBITED ACCORDING TO THE INVENTION

According to the invention, double stranded DNA oligonucleotides may be used to inhibit any transcriptional factor which influences transcription by binding to controlling elements of a gene. In particular embodiments of the invention, the transcriptional factor acts to increase transcription of a gene, for example, by binding

to a promoter/enhancer element. In alternate embodiments of the invention, the transcriptional factor acts to repress transcription of a gene. Examples of transcriptional repressors include, but are not limited to, the bovine papillomavirus E2 gene. Examples of transcription factors which increase transcription include, but are not limited to, those listed in Table I.

TABLE I

Transcription Factor	Reference
lymphoid-specific	Muller et al., 1988, Nature 336:544-551 Scheidereit et al., 1988, Nature 336:551-557
LF-B1 (liver-specific)	Frain et al., 1989, Cell 59:145-157
Pituitary Specific	Bodner et al., 1988, Cell 55:505-518 Ingraham et al., 1988, Cell 55:519-529
Active on EGF Receptor Gene	Kageyama et al., 1988, Proc. Natl Acad. Sci. USA 85:5016-5020
Fos protein	Lucibello et al., 1988, Oncogene 3:43-52
v-jun protein	Bos et al., 1988, Cell 52:705-712
PEA 1	Wasylyk et al., 1988, EMBO J. 1:2475-2483 [&]
EF-C	Ostapchuk et al., 1989, Mol. Cell. Biol. 9:2787-2797

According to preferred embodiments of the invention, oligonucleotides may be used to inhibit the function of transcription factors which are capable of increasing transcription by competing with native DNA binding sites for transcription factor, thereby effectively inhibiting the transcription of a gene (or genes) which is (are) influenced by said transcription factor. For example, and not by way of limitation, double stranded DNA oligonucleotides which bind to a transcription factor which induces the transcription of a viral gene may compete with viral promoter/enhancer elements for transcription factor binding and thereby effectively inhibit the transcription of viral gene sequences.

In alternate embodiments of the invention, double stranded DNA oligonucleotides may be used to inhibit the function of transcription factors which are capable of repressing transcription by competing with native DNA binding sites for the repressor, thereby effectively increasing the transcription of a gene (or genes) which is (are) influenced by said transcription factor. For example, and not by way of limitation, double stranded DNA oligonucleotides which bind to a viral repressor protein which normally renders a viral gene or genes functionally inactive may be used to activate the expression of these viral genes in a controlled manner; this method may prove useful in the study of latent viral infection in animal models.

Importantly, a particular transcription factor need not be characterized in order to be inhibited according to the present invention. It would be sufficient for a DNA sequence which influences transcription to be identified. For example, the transcription of a particular gene under study may be found to be controlled by a mechanism which includes the presence of a particular DNA sequence; such a sequence might be identified by studying

mutations of the gene and its surrounding DNA sequences. Mutation of a sequence important in promoting transcription (a promoter element) may be found to result in a relative decrease in the transcription of a particular gene (either the gene naturally associated with the promoter element or a reporter gene put under the control of the promoter element). Also, DNA sequences which bind to potential transcription factors may be identified by footprinting techniques or gel retardation analysis using standard techniques known in the art. The DNA sequence of the binding site may then be determined using standard sequencing techniques (for example, Sanger et al., 1979, Proc. Natl. Acad. Sci. U.S.A. 72:3918-3921).

DNA sequences which bind to repressor transcription factors may be identified in an analogous manner.

5.2. IDENTIFICATION OF OLIGONUCLEOTIDES THAT MAY BE USED TO INHIBIT TRANSCRIPTION FACTOR BINDING TO CONTROL ELEMENTS

Oligonucleotides which may be used to inhibit transcription factor binding to control elements may be identified by determining whether said oligonucleotides (a) bind to said transcription factor and/or (b) inhibit the function of said transcription factor.

Oligonucleotides may be tested for an ability to bind to a transcription factor by any method known in the art, including, but not limited to, the following.

If a transcription factor (TF) has been characterized and purified, the capability of an oligonucleotide may be tested directly for binding to the TF. For example, TF may be immobilized, and then exposed to labeled oligonucleotide, upon which selective retention of labeled oligonucleotide to TF could be measured.

Whether a TF has or has not been characterized, but it has been shown, by DNase footprinting analysis (Galas and Schmitz, 1978, Nucl. Acids Res. 5:3157-3170; Bos et al., 1988, cell 52:705-712), to bind to a particular DNA sequence, the binding capability of an oligonucleotide may be tested indirectly. For example, oligonucleotide may be included in the reaction in which TF (in purified or unpurified form) is allowed to bind to DNA; the oligonucleotide may be observed to competitively inhibit the binding of TF to its target sequence, thereby diminishing the appearance of a clear "footprint."

Alternatively, characterized or uncharacterized, purified or unpurified TF may be tested for the ability to bind an oligonucleotide using gel retardation analysis (Barberis et al., 1987, cell 50:347-359). For example, an oligonucleotide could be exposed to purified TF or, alternatively, TF as found in a mixture (e.g. a nuclear extract) under conditions which may allow binding of TF to the oligonucleotide. When subjected to polyacrylamide gel electrophoresis, the mobility of oligonucleotide bound to TF would be expected to be retarded relative to the mobility of unbound oligonucleotide.

Alternatively, oligonucleotides may be tested for the ability to inhibit the function (e.g. as inducer or repressor) of said transcription factor. Oligonucleotides may be evaluated using transcription systems in vitro or in vivo which comprise a control element which is believed to interact with a transcription factor and which controls the expression of a test gene.

For example, and not by way of limitation, in a specific embodiment of the invention which is exemplified in Section 6, infra, the ability of an oligonucleotide to inhibit transcription of the adenovirus E1B gene may be tested in vitro as follows. Nuclear extracts may be prepared from actively growing cells as described in

Section 6.1.3., infra. Transcription mixtures may contain the following: about 0.5-1.2 μg of DNA comprising a test gene under the control of an element which is believed to bind to a transcription factor in a final concentration of 226 mM HEPES pH 7.9, 48 mM KCl, 6 mM $MgCl_2$, 9.6% glycerol, 0.1 mM EDTA, 0.6 mM of ATP, GTP, CTP and UTP, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride, and competitor oligonucleotide (0.1 to 1.0 μg/reaction; preferably, a range of amounts are tested). Reaction may be started by addition of extract (generally about 1-2 μg protein/μl nuclear extract) incubated 30-90 minutes at 30°C, and terminated by addition of a mixture of 175 μg of 200 mM NaCl,20 mM EDTA and 1% sodium dodecyl sulfate, 20 μg purified yeast tRNA, followed by extraction with 100 μg phenol and 100 μg chloroform-isoamyl alcohol (19:1) to each tube. The oligonucleotides in the aqueous phase may be precipitated with 0.5 M NH_4 acetate and 3 volumes of ethanol, resuspended in 200 μg 0.3 M Na acetate pH6, reprecipitated with 3 volumes of ethanol and dried in a vacuum centrifuge.

The mRNA products of transcription in this, or any transcription assay, may be analyzed by any method known in the art, including, but not limited to, Northern blot analysis and/or quantitative hybridization (e.g. hybridization of labeled mRNA to DNA immobilized on filters). In a preferred embodiment of the invention, a primer extension assay, such as that developed with E1B for analysis of transcriptional factors (see Section 6.1.2. and 6.2.1., infra) may be used; in particular, the residue from the aqueous phase of the transcription reaction may be dissolved in 10 μg of 0.25 M KCl in TE buffer containing 0.17-0.24 ng of oligonucleotide primer 5'-phosphorylated with [^{32}P]-ATP and annealed at 65° for about 30 minutes. The solution may then be cooled and the primer extended by incubation for about 30 minutes at 37°C in a solution

containing 14 mM tris buffer pH8, 7 mM $MgCl_2$, 3.5 mM DTT, 0.2 mM each of dATP, dGTP, dCTP and DTTP, 7 μg/ml of actinomycin D and 0.085 U/μl Maloney murine leukemia virus reverse transcriptase in a final volume of about 35 μl. Following the reaction, the nucleic acid may be precipitated with 0.3 M Na acetate and 3 volumes of ethanol, dried, and dissolved in 10 μg buffered formamide containing bromphenol blue and xylene cyanol, then subjected to polyacrylamide gel electrophoresis at 300-400V in a 8 M urea, 10% acrylamide, 0.3% bis-acrylamide gel. Bands may be cut from dried gels and quantified by liquid scintillation counting.

As another example, the activity of a TF may be evaluated in vitro in a system which comprises the TF and a transcription template that includes a reporter gene under the control of a promoter which binds to said TF. The effect of test oligonucleotides on reporter gene expression may be expected to reflect the effectiveness of the oligonucleotide in inhibiting TF binding. For example, and not by way of limitation, an in vitro system may be utilized in which HIV tat protein is the TF and the transcription template is a reporter gene, such as the gene encoding chloramphenicol acetyltransferase (CAT), under the control of the HIV-1 LTR promoter. In such a system, double stranded oligonucleotides comprising one or more SP1 or NF kappa B sites, or both, may inhibit tat activity.

Alternatively, transcription may be carried out in isolated nuclei or whole cells which comprise the control element which is believed to bind to transcription factor, such that inhibition of TF binding to the control element and consequent inhibition of TF function is detectable. For example, the amount of RNA transcribed from a gene controlled by said control element may be measured in the presence or absence of oligonucleotide in pulse-labeling experiments of cells or isolated nuclei.

Alternatively, a protein product of a gene controlled by said control element may be monitored in the presence or absence of oligonucleotide; proteins suitable may include, but are not limited to, standard reporter genes such as chloramphenicol/acetyltransferase, β-galactosidase, luciferase, etc.

For example, a transcription template comprising a reporter gene and a control element may be transfected into cells which contain a transcription factor of interest. The effect on reporter gene expression of exposing such transfected cells to oligonucleotides of the invention may be used as a measure of the effectiveness of the oligonucleotides in entering the cells and inhibiting TF binding. Any suitable reporter gene may be used. The term "reporter gene," as used herein, refers to any detectable gene product; reporter gene products which are easily and inexpensively detected are preferred. It may be desirable, under certain circumstances, to use a reporter gene that encodes a product for which a highly sensitive assay is available. For example, and not by way of limitation, a highly sensitive radioimmunoassay is available for human growth hormone (HGH). Accordingly, a specific assay system, such as HeLa cells which express tat protein transfected with a transcription template that comprises the gene for HGH under the control of the HIV-1 LTR, may be used to test the efficacy of oligonucleotides inhibiting tat binding in a non-limiting embodiment of the present invention.

Oligonucleotide sequences which may represent TF binding sites may be identified by techniques including mutational analysis, footprinting studies, and gel retardation analysis as described above. Oligonucleotide sequences which have been associated with TF binding and HIV-1 transcriptional regulation, and which may be used

according to the invention, are presented in Table II. Binding site sequences which are as yet to be characterized are also provided for by the invention.

TABLE II

OLIGONUCLEOTIDE SEQUENCES USEFUL FOR INHIBITING HIV-1 TRANSCRIPTION

	REGION	REFERENCE
GGGACTTCC	Kappa enhancer	Wu et al., 1988, EMBO J. 7:2117-2130
CTCTCTGG	TAR	"
GGGCGG	SP-1	Gidoni et al., 1984, Nature 312:409-413
TGAGTCAG	AP-1	Angel et al., 1987 Cell 49:729-739
C/GTGACTC/AA	AP-1	Lee et al., 1987, Cell 49:741-752

5.3. OLIGONUCLEOTIDES OF THE INVENTION

Oligonucleotides may be synthesized using any technique used in the art. The present invention construes oligonucleotides to mean a series of nucleotides linked together, and includes nucleotides linked in a standard 5'-3' phosphodiester linkage and also molecules comprising a methylated nucleotide or nucleotide similarly modified, or any nucleoside analogue or enantiomer, or nucleosides joined by phosphorothioate linkage or molecules which comprise a variety of chemical linkages (e.g.

phosphodiester and phosphorothioate). In preferred embodiments of the invention, an oligonucleotide comprises at least one phosphorothioate linkage. Phosphorothioate modification of an oligonucleotide has been observed to enhance TF inhibition, and represents a preferred embodiment of the invention. Furthermore, as shown in example section 7, infra, phosphorothioate-linked oligonucleotides are able to reach higher concentrations in cells compared to phosphodiester-linked oligonucleotides.

In a preferred embodiment, phosphorothioate-linkages may be enzymatically introduced into double-stranded oligonucleotides of the invention. For example, and not by way of limitation, phosphorothioate linked oligonucleotides may be prepared by a reaction comprising commercially available alpha-thio-nucleotides and primers which include putative TF-binding sequences, utilizing polymerase chain reaction (PCR) technology (Saiki et al., 1985, Science 230:1350-1354).

The oligonucleotides of the invention comprise at least a portion which is double stranded, and include oligonucleotides with blunt (double-stranded) ends as well as oligonucleotides with single stranded "overhangs", in which the ends of the molecule are extensions of single-stranded nucleotide sequence beyond a double stranded nucleotide sequence beyond a double stranded region. It has been observed that oligonucleotides which comprise a single-stranded "overhang" are more effective in inhibiting TF binding, and accordingly represent preferred embodiments of the invention.

The oligonucleotides of the invention comprise one or, preferably, more than one binding site for a transcription factor. Oligonucleotides of the invention may also comprise binding sites for more than one species

of transcription factor; this may be particularly useful if two transcription factors function coordinately to control transcription of a gene.

It may be desirable for the oligonucleotides of the invention be linked to molecules which may aid the ability of the oligonucleotides to physically reach the transcriptional apparatus. Examples may include molecules with hydrophobic regions which may facilitate the penetration of the oligonucleotide through the cell membrane. Alternatively, the oligonucleotides may be linked to a nuclear localization signal (such as is utilized by SV40). Alternatively, oligonucleotides of the invention may be targeted toward a particular cell type or tissue (e.g. virus-infected cells) by an antibody specific for that cell type or tissue; oligonucleotide may be released from the antibody and then function to inhibit transcription in a particular cell type or tissue. Further, oligonucleotides may be comprised in liposomes or microcapsules, which also offers the advantage of preventing degradation of the oligonucleotides prior to cellular uptake.

5.4. UTILITY OF THE INVENTION

The present invention may be used to either increase or decrease the transcription of a gene or genes, the transcription of which is regulated by the interaction of a control element of the gene or genes and a transcription factor. According to the invention, oligonucleotides are used to inhibit the binding of the transcription factor to its control element. If the transcription factor normally acts to increase transcription, then transcription may effectively be decreased by competing oligonucleotides. If the

transcription factor normally acts to repress transcription, then transcription may be effectively increased by competing oligonucleotides.

According to one specific embodiment of the invention, the transcription factor to be inhibited is SP1. According to other specific embodiments of the invention, the transcription factor is the HIV-1 tat protein, or, alternatively, the HTLV-I tax protein, or NF-kappa B.

Transcription factors may specifically influence the expression of some genes, but not others; for example, some TFs specifically influence the transcription of viral genes, and others specifically influence the transcription of genes in certain tissues (including, but not limited to, pituitary or lymphoid cells). The specificity of TFs enables the manipulation of the transcription of genes of interest via the specific inhibition of TF binding by oligonucleotides.

For TFs which are not optimally specific, it may be desirable to direct oligonucleotides to a subpopulation of cells which utilize the TF of interest. For example, a cellular TF may induce the transcription of viral as well as cellular genes; if oligonucleotide were supplied to all cells in the body of an organism, transcription of viral and cellular genes would be inhibited in a manner potentially harmful to the organism as a whole. If, however, oligonucleotides were delivered only to virus infected cells (e.g. via antibody to a viral antigen present on the surface of infected cells, or an antibody-targeted liposome or microcapsule), then only infected cells would be affected.

The use of oligonucleotides which comprise double-stranded regions offers advantages over the use of other competitive oligonucleotides such as antisense RNA. Such advantages include increased stability, particularly in the case of oligonucleotides comprising a

phosphorothioate linkage and the fact that lower concentrations of oligonucleotide may be required to effectively compete with transcription factors (present in low concentration) as compared to the concentration required to compete with mRNA (present in higher concentrations), (See Section 6.3., infra).

The present invention may be utilized to control the transcription rates of any gene influenced by transcription factor binding, including cellular genes and viral genes. Clinical applications may include, but are not limited to, decreasing the imbalance of expression of globin genes in thalassemia, inhibiting the transcription of oncogenes in the treatment or prevention of malignancy inhibiting the production of growth hormone in acromegaly and inhibiting the transcription of oncogenes in the treatment or prevention of malignancy. The present invention also provides for treatment of a wide variety of viral diseases, including, but not limited to, AIDS, HTLV-I infection, and papillomavirus infection.

6. EXAMPLE: INHIBITION OF IN VITRO TRANSCRIPTION BY SPECIFIC DOUBLE-STRANDED OLIGONUCLEOTIDES

6.1. MATERIALS AND METHODS

6.1.1. PLASMID CONSTRUCTIONS

The E1B transcriptional unit was the XbaI-SacI fragment, nucleotides 1336 to 1767 of the adenovirus-2 genome inserted into a PUC18 vector (Wu, supra and Gineras et al., 1982, J. Biol. Chem. 257:13475-13491). A single stranded oligonucleotide complementary to nucleotides 1730 to 1750 was used for primer extension analysis of E1B transcription. The vector and primer were gifts from M. Schmidt and A. Berk, Molecular Biology Institute, University of California, Los Angeles.

The pDUG plasmid consists of the E fragment of a BalI digest of the adenovirus-2 genome inserted into a PHC 314 vector. The E fragment contains the major late promoter of the adenovirus but no SP1 binding site (Leong et al., 1988, Mol. Cell. Biol. 8:1785-1774.

6.1.2. OLIGONUCLEOTIDE SYNTHESIS

Complementary 14 mers identical to the ones used by Kadonaga, J. T. and Tjian, R., 1986, Proc. Natl. Acad. Sci. (USA), 83:5889-5893 for purification of the SP1 transcriptional factor were chemically synthesized by D. Glick, Department of Biochemistry, University of California, Los Angeles. Additional oligonucleotides were synthesized by J. Tomich, Division of Genetics, Children's Hospital of Los Angeles, or the Microchemical Core Facility at the Norris-USC Comprehensive Cancer Center. Complementary 28 mers containing the SP1 binding site were synthesized. One set, 17/19, contained no 5-methylcytosine (5-mCyt) residues, another set, 21/25, contained a single 5-mCyt residue within the SP1 binding site. A third set, 18/20, had every cytidine residue methylated. To determine the optimal conditions for inhibition of transcription, double stranded sets of oligonucleotides containing one, two or three SP1 sites were synthesized. Some oligonucleotide sets were made with both blunt ends and 4 nucleotide complementary overhangs. To determine the effect of unrelated or low SP1 affinity sequences on inhibition of E1B transcription, oligonucleotides were synthesized corresponding to binding sites for AP2, a TATA box and a low affinity binding sequence for SP1 from SV40 (Kadonaga, J. T. and Tjian, R., 1986, Proc. Natl. Acad. Sci. (USA), 83:5889-5893). An oligonucleotide containing both an SP1 site and a TATA box separated by the same number of nucleotides as in the E1B transcriptional unit was synthesized to look for a possible interaction between

the two factors. Phosphorothioate analogues of the blunt ended oligonucleotide set containing 2 SP1 sites, SP1X2B, and a self-complementary 26 mer containing the PvuI restriction site were synthesized by hydrogen phosphonate chemistry on an Applied Biosystems DNA synthesizer and by single step sulfurization following chain assembly. All oligonucleotides were purified and annealed as described (Harrington et al., 1988, Proc. Natl. Acad. Sci. (USA), 85:2066-2070).

The sequence of the E1B transcriptional unit, 14 mers, 28 mers and primer are shown in Fig. 1. The SP1 binding site and TATA box are underlined. Transcription begins at residue +1 (Wu, supra). The sequences of other oligonucleotides are listed in Table III.

TABLE III

SEQUENCES OF COMPETING OLIGONUCLEOTIDES

Name	Sequence
SP1S	GATCGGGGCGGGGC CCCCGCCCCGCTAG
SP1B	GATCGGGGCGGGGC CTAGCCCCGCCCCG
SP1L	GATCGGGGCGGAGA CTAGCCCCGCCTCT
TATA	CTGCATAAATAAAAAAAA GACGTATTTATTTTTTT
AP2	GCCTGGGGAGCCTGGGGAGC CGGACCCCTCGGACCCCTCG
SP1TATA	GGGGCGGGGCTTAAAGGGTTTTTTTTATTTAT CCCCGCCCGGAATTTCCCAAAAAAAATAAATA
SP1X2S	GATCGGGGCGGGGCGGGGGGCGGGGC CCCCGCCCCGCCCCCCGCCCCGCTAG
SP1X2B	GATCGGGGCGGGGCGGGGGGCGGGGC CTAGCCCCGCCCCGCCCCCCGCCCCG
SP1X3S	GATCGGGGCGGGGCGGGGGGCGGGGCAAGGGGCGGGGC CCCCGCCCCGCCCCCCGCCCCGTTCCCCGCCCCGCTAG

6.1.3. IN VITRO TRANSCRIPTION

MOLT 4 and BJAB human lymphoid cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum; P3X mouse plasmacytoma cells were grown in DMEM medium containing 10% horse serum and HeLa cells were grown in spinner bottles in MEM containing 5% newborn calf serum.

All media contained 50mg/liter gentamicin. Cells were harvested in log phase of growth, rapidly chilled with frozen phosphate-buffered saline (PBS), centrifuged and washed in ice cold PBS. Nuclear extracts were prepared as described, dialyzed for 4-5 hours in buffer D (Dignam et al., 1983, nucleic Acids Res. 11:1475-1489), clarified by centrifugation and frozen in liquid nitrogen. The extracts contained 3-5 mg/ml protein. They were stable for several months stored in small aliquots at -70°C.

Twenty-five μl transcription mixtures contained 0.5-1.2 μl E1B plasmid DNA or pDUG plasmid DNA in a final concentration of 26 mM HEPES pH 7.9, 48 mM KCl, 6 mM $MgCl_2$, 9.6% glycerol, 0.1 mM EDTA, 0.6 mM each of ATP, GTP CTP and UTP, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonylfluoride along with varying amounts of competitor oligonucleotide. The reaction was started by addition of the extract and incubated 30-90 minutes at 30°C. It was terminated by addition of a mixture of 175 μl of 200 mM NaCl, 20 mM EDTA and 1% Na dodecyl sulfate, 20 μg purified yeast t-RNA, and extracted with the addition of 100 μl phenol and 100 μl chloroform-isoamyl alcohol (19:1) to each tube. The oligonucleotides in the aqueous phase were precipitated with 0.5 M NH_4 acetate and 3 volumes of ethanol, resuspended in 200 μl 0.3 M Na acetate pH 6, reprecipitated with 3 volumes of ethanol and dried in a vacuum centrifuge.

For analysis of the mRNA transcribed, the residue from the aqueous phase was dissolved in 10 μl of 0.25 M KCl in TE buffer containing 0.17-0.24 ng of the 20 nucleotide primer 5'-phosphorylated with [^{32}P] ATP and annealed at 65° C for 30 minutes. The solution was cooled and the primer extended by incubation for 30 minutes at 37 C in a solution containing 14 mM Tris buffer pH8, 7 mM $MgCl_2$, 3.5 mM DDT, 0.2 mM each of dATP, dGTP, dCTP and TTP, 7 μg/ml actinomycin D and 0.085 U/μl MMLV referse

transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, USA) in a final volume of 35 µl. The nucleic acid was precipitated with 0.3 M Na acetate and 3 volumes of ethanol, dried, and dissolved in 10 µl buffered formamide containing bromphenol blue and xylene cyanol. They were electrophoresed at 300-400 V in a 8 M urea, 10% acrylamide, 0.3% bis-acrylamide gel. Transcription of the E1B template resulted in a 50 nucleotide band. The bands were cut from the dried gels and quantified by liquid scintillation counting.

The degree of polymerization was studied by incubation of [^{32}P]-labelled oligonucleotides under the same conditions as for in vitro transcription. After precipitation with ethanol, the dried residue was dissolved in running dye and electrophorsed under non-denaturing conditions at 300-400 V in a 10% acrylamide, 0.3% bis-acrylamide gel. All bands were cut from the dried gels and quantified by liquid scintillation counting.

6.2. RESULTS

6.2.1. IN VITRO TRANSCRIPTION

This primer extension assay with E1B was developed for analysis of transcriptional factors in nuclear extracts by M. Schmidt, Molecular Biology Institute, University of California, Los Angeles (Schmidt, M. and Berk, A., unpublished observations). Nuclear extracts of lymphoid cells were used in this study since we were characterizing specific nuclear binding factors in these cells (Peterson, supra). Preliminary experiments showed that extracts from Molt 4, BJAB or P3X cells could transcribe the E1B template with formation of the expected 50 nucleotide band. The amount of transcript increased with extract concentration, time to 90 min. and template concentration to 21 µl/ml (11 nM). With some nuclear extracts, transcription was lower at a template

concentration of 40 μg/ml. Two μg/ml α-amanitin (Sigma, St. Louis, MO) inhibited the transcription indicating that it was dependent on RNA polymerase II. $MgCl_2$ concentrations were optimal between 6 and 7.5 mM with different extracts. Spermidine (1-4 mM) inhibited transcription and decreased the effect of α-amanitin. Addition of 4.8% polyethylene glycol 20,000 MW (Baker, Phillipsburg, NJ) or 2% polyvinyl alcohol 10,000 MW (Sigma) increased transcription with some extracts. Preincubation with template before addition of NTPs did not increase the level of transcription. In competition experiments, preincubation of the oligonucleotides with the nuclear extract prior to addition of the E1B plasmid did not increase the degree of inhibition.

6.2.2. INHIBITION OF SP1-BINDING OLIGONUCLEOTIDES

Formation of the 50 nucleotide transcript was inhibited by various double stranded oligonucleotides. The percentages of control transcription at various concentrations of competing oligomers were plotted on a semilog scale and analyzed by first and second order regression lines. A radioautograph of a typical experiment with a Molt 4 nuclear extract is shown in Fig. 2.

TABLE IV

INHIBITION OF E1B TRANSCRIPTION BY DOUBLE STRANDED OLIGONUCLEOTIDES

	Percentages of control transcription			
	20%	50%		
	µg/25µl	µg/25µl	µM	molar ratio
One SP1 site				
SP1S	0.60	0.20	0.87	83
SP1B	1.10	0.20	0.87	83
SP1L	1.58	0.64	2.80	260
17/19	0.68	0.22	0.48	45
21/25	0.80	0.17	0.37	35
18/20	0.85	0.34	0.74	70
Two SP1 sites				
SP1X2S	0.33	0.15	0.35	33
SP1X2B	0.30	0.07	0.16	16
Three SP1 sites				
SP1X3S	0.20	0.05	0.07	7
Others				
TATA	1.38	0.61	2.05	200
AP2	1.37	0.65	1.97	190
SP1TATA	1.13	0.37	0.70	67

The amount of competitor oligonucleotide required to inhibit to 20% and 50% of control was determined from regression lines using Sigmaplot statistical software. The amount required for 50% inhibition is also shown as oligonucleotide concentration and the molar ratio of oligonucleotide to template DNA.

Table IV shows the concentrations of the various oligonucleotides needed to decrease E1B transcription to 20% and 50% of the control without competitor oligonucleotides. An IC20 value gives an approximate concentration needed to achieve maximal inhibition. The IC50 is also expressed as the molar ratio of oligonucleotide to template DNA.

The IC50 was 0.87 μM for SP1S and SP1B, 14 mers with sticky and blunt ends. An oligonucleotide with a low affinity SP1 binding site, SP1L, required an IC50 of 2.80 μM. The IC50 of the unmethylated 28 mer, 17/19, was 0.48 μM. The addition of a single methylated cytosine in 21/25 did not affect transcription of E1B, while the completely methylated 28 mer, 18/20, had an IC50 of 0.74 μM. Thus, the effect of a single methylation was minimal on the ability of the synthetic oligonucleotides to compete in this transcription assay. A completely methylated oligonucleotide had a decreased ability to compete for SP1.

To determine whether an oligonucleotide with more than one SP1 site on the same side of the DNA duplex would be more effective at competing for SP1 factors, oligonucleotides with two and three SP1 sites separated by 12 nucleotides were synthesized (Fig. 3). IC50 concentrations for the sticky ended oligonucleotides with one, two or three SP1 sites were 0.87, 0.35 and 0.07 μM, respectively. IC50 concentration for the blunt ended oligonucleotide with two SP1 sites was 0.16 μM. Inhibition of transcription to 20% of control followed the same pattern.

When the concentration of E1B template was varied at a constant concentration of competing oligonucleotide, the percentage inhibition of transcription changed. Greater inhibition of transcription was seen at sub-saturating concentrations of E1B template than at saturating concentrations. This suggests that molar ratios

are important in determining the efficacy of these double stranded oligonucleotides with multiple SP1 sites. A molar ratio of only 7 was needed for 50% inhibition with the oligonucleotide containing 3 SP1 sites compared to 83 for the oligonucleotides with only one SP1 site.

To assess the specificity of the inhibition, oligonucleotides were synthesized that contained a TATA binding site or an AP2 site (Mitchell et al., 1987, Cell, 50:847-861). These oligonucleotides competed for binding factors much less efficiently. IC50 concentrations ranged from 1.97 μM to 2.05 μM, approximately threefold greater than the amount required for the oligonucleotides with high affinity SP1 sites. The competition by the AP2 oligonucleotide is consistent with the interactions reported between SP1 and AP2 with DNAase protection assays (Mitchell et al., 1987, Cell, 50:847-861).

Another oligonucleotide, SP1TATA, contained a single SP1 binding site and a TATA binding site with the same spacing as in the E1B promoter. This oligonucleotide was only slightly better inhibitor than an oligonucleotide with only an SP1 site.

Oligonucleotides with blunt ends or with four base complimentary overhangs showed differences in the shape of the inhibition curves, but similar IC50 and IC20 values. During incubation with nuclear extracts, the sticky ended oligonucleotides might form higher molecular weight structures more readily than the blunt ended ones. Such higher polymers might have greater affinity for SP1 (Kadonaga, J. T. and Tjian, R., supra). Polymerization was tested directly with end labelled oligonucleotides. The amount of higher molecular weight structures formed was less than 10% of the total, with both sticky and blunt oligonucleotides after 90 minutes incubation with MOLT 4 nuclear extracts. During this incubation with blunt and sticky ended oligonucleotides were degraded to 23% and to

55% of their original amount, respectively. To show the specificity of inhibition, we tested the effect of unrelated oligonucleotides and the upper and lower strand of the 14 mer SP1S oligonucleotide. Transcription was 140% of control with 0.4 μg/25 μl and 91% of control when 1.0 μg/25 μl of a double stranded PvuI linker (N.E. Biolabs), 5'-TCGCGATCGCGA-3', was added. Addition of 0.5 μg/25 μl or 1.0 μg/25 μl of the lower strand 14 mer (Fig. 1) caused a decrease to 98% and 78% of the control, respectively. In contrast, 0.5 μg/25 μl or 1.0 μg/25 μl of the upper strand of the 14 mer caused a decrease to 71% and 35% of the control, respectively. Thus, the presence of a double stranded oligomer without a GC box did not compete for SP1, while the single stranded oligonucleotide containing a GC box did compete for SP1 binding at high concentrations. This competition by the guanine rich strand of SP1 is consistent with a report that methylation protection by SP1 occurs only on the guanine rich strand (Gidoni et al., 1984, Nature, 312:409-413).

We also tested oligonucleotides that compete for E1B transcription in assays with the E fragment of a BalI digest of the adenovirus 2 genome. This sequence contains the major late promoter of the adenovirus and no SP1 site (Leong, 1988, supra). Transcription with Molt 4 and HeLa nuclear extracts was inhibited only 20% with concentrations of competing oligonucleotide up to 1.5 μg/μl. Transcription was actually enhanced with low concentrations of the competing oligonucleotide.

We synthesized and annealed a set of phosphorothioate linked oligonucleotides with the same sequences as SP1X2B, the blunt ended set with 2 LSP1 sites. Transcription was only 5% of control values with concentrations of 0.04 μg/25 μl (0.09 μM) or greater of the phosphorothioate derivative while SP1x2B had an IC20 of 0.30 μg/25 μl. Thus, the double stranded phosphorothioate

was a more effective inhibitor than SP1X2B. A control 26 mer phosphorothioate containing a PvuI site did not inhibit E1B transcription at concentrations of 1 μg/25 μl.

6.3. DISCUSSION

In vitro assays have been used to define the factors necessary for transcription of specific genes by RNA polymerases II and III (Gidoni et al, 1984, supra; Chodoch et al., 1986, Mol. Cell. Biol., 6:4723-4733; Hawley, D. K. and Roeder, R. G., 1985, J. Biol. Chem. 260:8163-8172 and Bieker et al., 1985, Cell 40:119-127). These experiments have identified the basic transcriptional factors needed for each polymerase as well as nuclear binding proteins that can increase or decrease the rate of initiation. If the factors for a particular gene are known, then it may be possible to inhibit or increase transcription by competing for the nuclear factors (Mulvihill, E. R. and Chambon, P., 1983, Nature, 301:680-686). Such an approach applied in an in vivo system may lead to a potential new therapy.

The studies reported here indicate that addition of short double-stranded oligomers containing the binding site for one of these factors, SP1, can inhibit in vitro transcription of E1B. Mutants lacking the SP1 site transcribe E1B at only 13 to 20% of basal levels, in vivo (Wu, 1987, supra). Inhibition of in vitro transcription to this level was achieved with concentrations less than 1 μM of the 14 and 28 mers containing 1 SP1 site. A single stranded oligonucleotide containing the GGGCGG sequence required higher concentrations to compete at the same levels as double stranded oligonucleotides.

A series of experiments were initiated to define the effects of the number of binding sites and sequence on the ability of double stranded oligonucleotides to inhibit transcription. Oligonucleotides with two SP1 sites inhibited transcription 50% with a molar ratio of

oligonucleotide to template that was approximately 3 times lower than an oligonucleotide with one SP1 site. The oligonucleotide with three SP1 sites inhibited 50% with a molar ratio that was approximately 11 times lower than the one with a single SP1 site. In contrast, an oligonucleotide set containing a SP1 site and TATA box was no more effective than one SP1 site. This data suggests that there are cooperative interactions between SP1 factors but not between SP1 and TATA.

Oligonucleotides with sticky ends were generally more effective in inhibiting transcription than blunt ends. This was not due to polymerization but may be due to better resistance to degradation.

Methylation of DNA has been proposed as an important component in the control of expression of certain eukaryotic genes (Yisraeli, J. and Azyl, M., 1984, DNA Methylation, 353-378). Several groups have shown that methylation of cytosine residues in GC boxes does not alter binding to SP1 (Harrington, 1988, supra, Hoeveler, A. and Doerfler, W., 1987, DNA 6:449-460; and Holler et al., 1989, Genes and Development 21:1127-1135). The present experiments assay confirm the lack of a direct effect of methylation of single residues on transcription regulated SP1 binding to the GC box. Multiple methylated sites had a slight effect on the ability of the oligonucleotide to bind to SP1. This may be due to greater steric hindrance.

Phosphorothioate oligonucleotides are more resistant to degradation by nucleases but this chemistry introduces an asymmetric center at each internucleotide linkage (Zon, G., 1988, Pharmaceutical Res., 5:539-549). In our assay, a phosphorothioate linked oligonucleotide was a better inhibitor of E1B transcription than its corresponding normal oligonucleotide. Thus, the

steroisomers do not appear to prevent SP1 binding. The improved effect may be due to resistance to nucleases present in cell extracts.

The use of double strandad oligonucleotides to control gene expression may present several advantages. Double stranded oligonucleotides are designed to bind nuclear binding factors rather than mRNA molecules. In most cases, there are few copies of transcriptional elements for a specific gene and few molecules of nuclear molecules of nuclear binding factors relative to the number of RNA transcripts. This advantage is illustrated by our data showing that the concentrations of double stranded oligonucleotides that inhibit transcription are 10 to 100 times lower than the reported concentrations of antisense oligonucleotides needed to block expression of various genes in vitro (Stein, 1988, supra). Our experiments have defined some specific parameters for double stranded oligonucleotides to achieve optimal inhibition of in vitro transcription of E1B. Similar experiments with more complex promoters may define interactions of their nuclear binding factors.

7. EXAMPLE: UPTAKE OF DOUBLE-STRANDED PHOSPHOROTHIOATE OLIGONUCLEOTIDES

It has been shown that double-stranded oligonucleotides with phosphorothioate linkages were taken up much more efficiently by MOLT 4 human leukemia cells in culture. As shown in Figure 4, the uptake of radiolabelled phosphodiester-linked SP1X2B oligonucleotides was significantly less than that of radiolabelled phosphorothioate-linked oligonucleotide SP1X2m which also has two SP1 sites. This difference in uptake may in fact reflect a difference in the stability of the oligonucleotides once inside the cell. This is supported by the observation that the uptake curves for both

oligonucleotides in Figure 4 were initially roughly parallel. Subsequently, however, the curve for phosphodiester-linked oligonucleotide uptake formed a plateau, whereas the curve for phosphorothioate-linked oligonucleotide continued to rise. This suggests that phosphorothiate-linked oligonucleotides had accumulated, but phosphodiester-linked oligonucleotides were degraded.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for controlling the expression of a gene comprising inhibiting the binding of a transcription factor to a transcriptional control element of the gene by competitively binding the transcription factor to a double-stranded oligonucleotide that comprises at least one phosphorothioate linkage.

2. The method of claim 1 which increases transcription of the gene.

3. The method of claim 1 which decreases the transcription of the gene.

4. The method of claim 3 in which the gene is a cellular gene.

5. The method of claim 3 in which the gene is a viral gene.

6. The method of claim 5 in which the viral gene is an adenovirus gene.

7. The method of claim 5 in which the viral gene is a papillomavirus gene.

8. The method of claim 5 in which the viral gene is a retrovirus gene.

9. The method of claim 8 in which the retrovirus is a causative agent of acquired immunodeficiency syndrome.

10. The method of claim 9 in which the retrovirus is human immunodeficiency virus 1.

11. The method of claim 10 in which the transcription factor is a product of the tat gene.

12. The method of claim 10 in which the transcription factor binds to the viral enhancer element.

13. The method of claim 10 in which the transcription factor binds to the negative regulatory region of the viral genome.

14. The method of claim 10 in which the transcription factor binds to an SP-1 site in the viral genome.

15. The method of claim 10 in which the transcription factor binds to the TAR region of the viral genome.

16. The method according to claim 3 in which the oligonucleotide comprises the sequence GGGCGG or at least a 4 bp subsequence thereof.

17. The method of claim 3 in which the oligonucleotide comprises the sequence GGACTTCC or at least a 4 bp subsequence thereof.

18. The method according to claim 3 in which the oligonucleotide comprises the sequence CTCTCTGG or at least a 4 bp subsequence thereof.

19. The method according to claim 9 in which the oligonucleotide comprises the sequence GGGCGG or at least a 4 bp subsequence thereof.

20. The method of claim 9 in which the oligonucleotide comprises the sequence GGACTTCC or at least a 4 bp subsequence thereof.

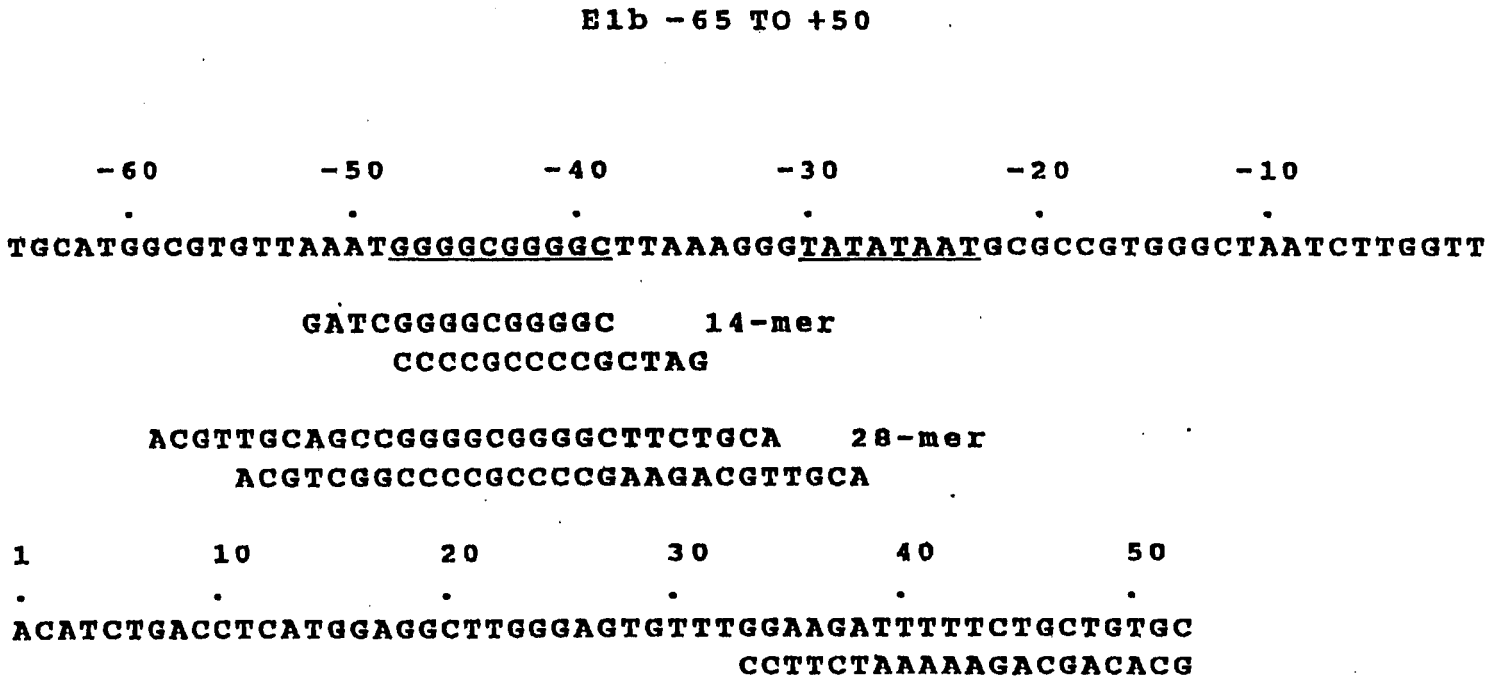
21. The method according to claim 9 in which the oligonucleotide comprises the sequence CTCTCTGG or at least a 4 bp subsequence thereof.

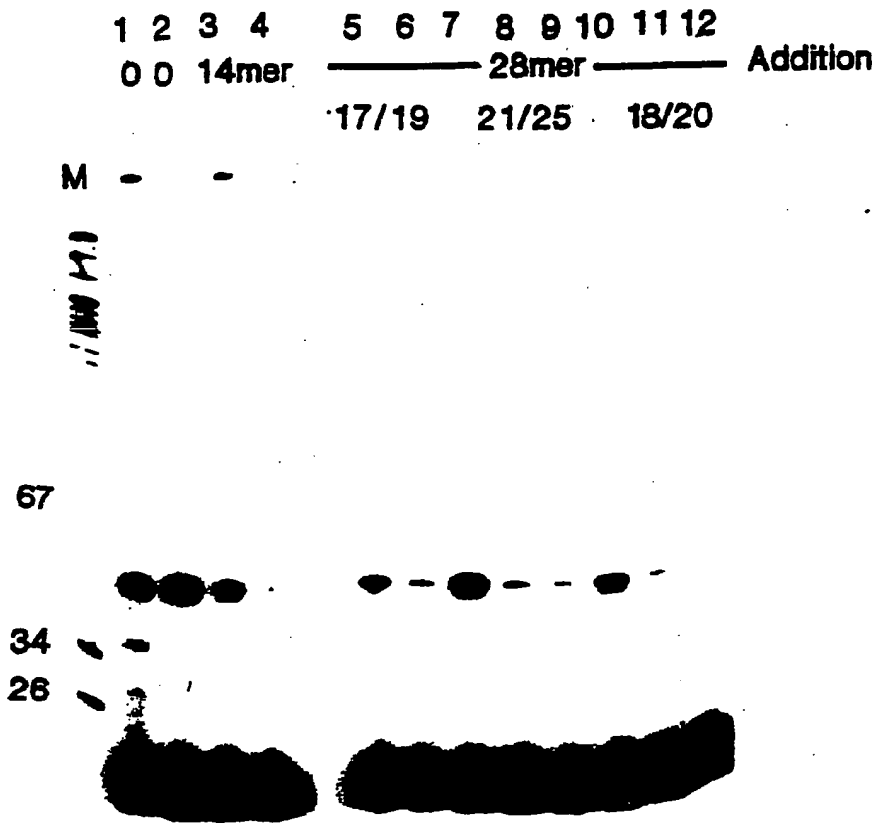
22. A composition for inhibiting the expression of a gene comprising a double-stranded oligonucleotide which comprises the sequence GGACTTCC or at least a 4 bp subsequence thereof and which comprises at least one phosphorothioate linkage.

23. A composition for inhibiting the expression of a gene comprising a double-stranded oligonucleotide which comprises the sequence CTCTCTGG or at least a 4 bp subsequence thereof and which comprises at least one phosphorothioate linkage.

24. A composition for inhibiting the expression of a gene comprising a double-stranded oligonucleotide which comprises the sequence GGGCGG or at least a 4 bp subsequence thereof and which comprises at least one phosphorothioate linkage.

FIG. 1

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0 0 14mer 28mer Addition
17/19 21/25 18/20
M
67
34
26

FIG. 2

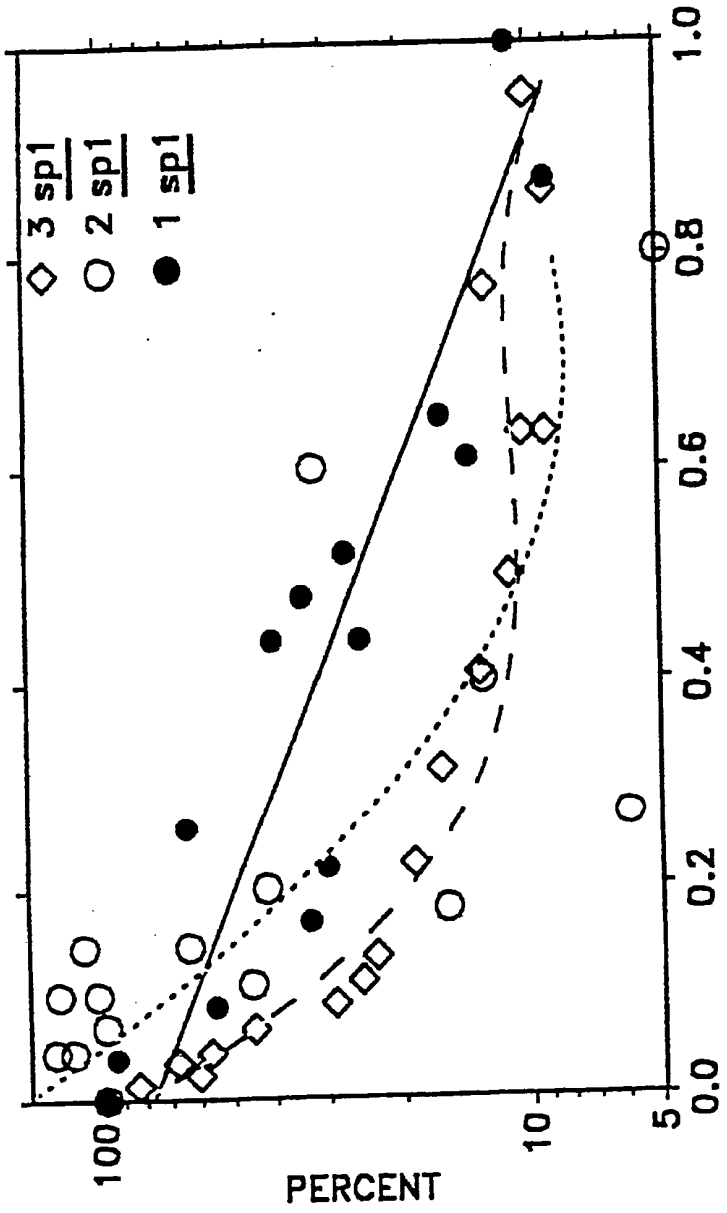

FIG. 3

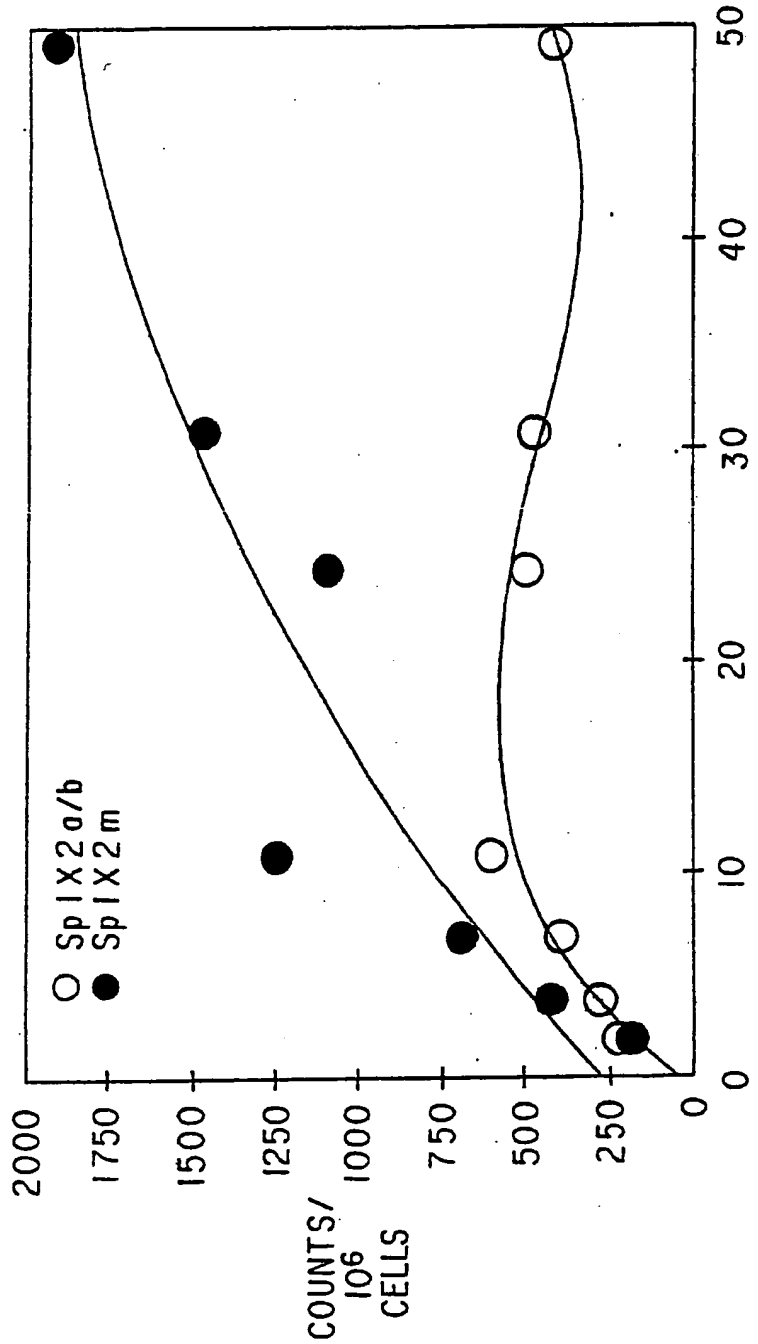
Sp I X 2 a/b
Sp I X 2 m
COUNTS/ 10^6 CELLS
2000
1750
1500
1250
1000
750
500
250
0
0
10
20
30
40
50
TIME (HRS)

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/00635

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

I.P.C.(5): C12Q 1/68; C07H 15/12; C12N 15/00

U.S.Cl. 435/6; 536/27; 935/77,78

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.	435/6; 536/27; 935/77,78

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

GenBank, EMBL, STN

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Nucleic Acids Research, Vol. 17, No. 21, issued 1989, Latchman et al, The Different Competitive Abilities of viral TAAGARAT Elements and cellular octamer Motifs, Mediate the induction of viral Immediate-Early Genes and the Repression of the Histone H2B Gene in Herps Simplex Virus infected cells" pages 8533-8542, see entiredocument.	1-5,8 6,7,9-21
X Y	Nature, Vol. 312, issued 29 November 1984, Gidoi et al, "Multiple specific contacts Between a Mammalian Transcription Factor and its cognate Promoters" pages 409-413, see entire document.	24 6,7,9-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

07 March 1991

Date of Mailing of this International Search Report

26 APR 1991

International Searching Authority

ISA/US

Authorized Officer

Mindy B. Fleisher

Form PCT/ISA/210 (second sheet) (rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	The EMBO Journal, Vol. 7, No. 7, issued 1988 Wu et al, "Purification of the Human immunodeficiency virus type 1 enhancer and TAR Binding Proteins EBP-1 and UBP-1" pages 2117-2129, see entire document.	22,23 6,7,9-21
Y	Cell, vol. 50, issued 03 July 1987, Lambert etal, "A Transcriptional Repressor Encoded by BPV-1 shares a common carboxy terminal Domain with the E2 transactivator" pages 69-78, see entire document.	6,7,9-21
Y	The EMBO Journal, Vol. 8, No. 3, issued 1989, Garcia etal, "Human immunodeficiency virus Type 1 LTR TATA and TAR Region Sequences Required for Transcriptional Regulation" Pages 765-778, see entire document.	6,7,9-21
Y	Science, Vol. 229, issued 05 July 1985, Arya etal, "Trans-Activator Gene of Human T-Lymphotrocpic Virus Type III (HTLV-III)" pages 69-73, see entire document.	6,7,9-21

US005157115A

United States Patent [19]

Taniguchi

[11] Patent Number: 5,157,115

[45] Date of Patent: Oct. 20, 1992

[54] REGULATION OF EXPRESSION

[76] Inventor: Tadatsugu Taniguchi, Mihogaoka 19, A-207, Ibaraki-shi, Osaka 567, Japan

[21] Appl. No.: 439,663

[22] Filed: Nov. 22, 1989

[30] Foreign Application Priority Data

Nov. 25, 1988 [GB] United Kingdom 8827592

[51] Int. Cl.[5] C07H 17/00

[52] U.S. Cl. 536/27

[58] Field of Search 536/27

[56] References Cited

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The European Search Report for the corresponding European application No. 89121590.7.

Primary Examiner—John W. Rollins

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[57] ABSTRACT

The inhibition or control of expression of IL-2 or IL-2α genes, and the inhibition or control of fundamental cellular processes responsible for retroviral replication, in particular HIV-I and HTLV-I DNA molecules, are disclosed. The invention is directed to nucleic acids or nucleic acid compositions which competitively bind regions of the IL-2 or IL-2α genes corresponding to their respective transcription factors. DNA or RNA fragments spanning the following regions in the IL-2 gene are shown to be effective; −195 to −204; −115 to −164; and −165 to −222. DNA or RNA fragments spanning the following regions in the IL-2α gene are shown to be effective: −62 to −71; and −32 to −86. Compositions comprising combinations of these fragments are also disclosed.

22 Claims, 3 Drawing Sheets

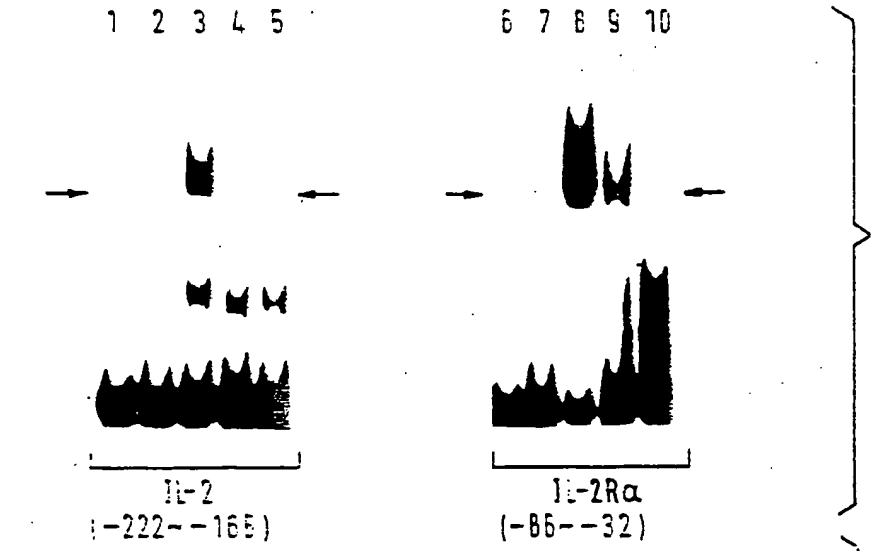


FIG.1a

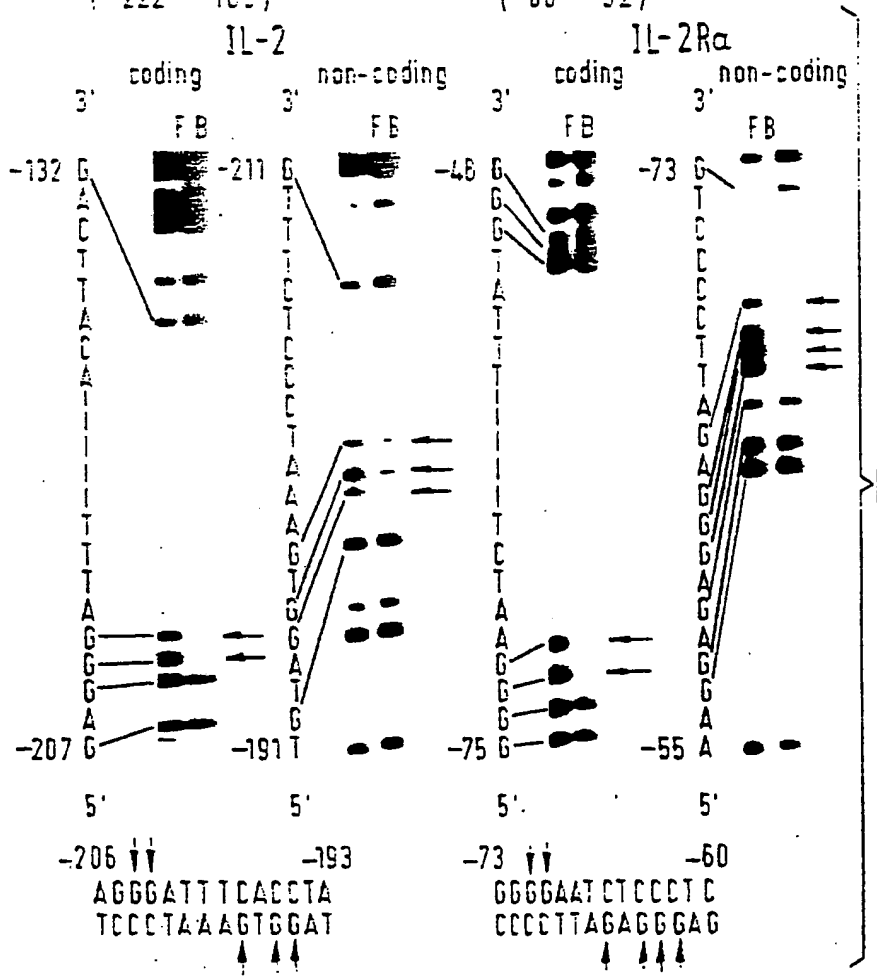
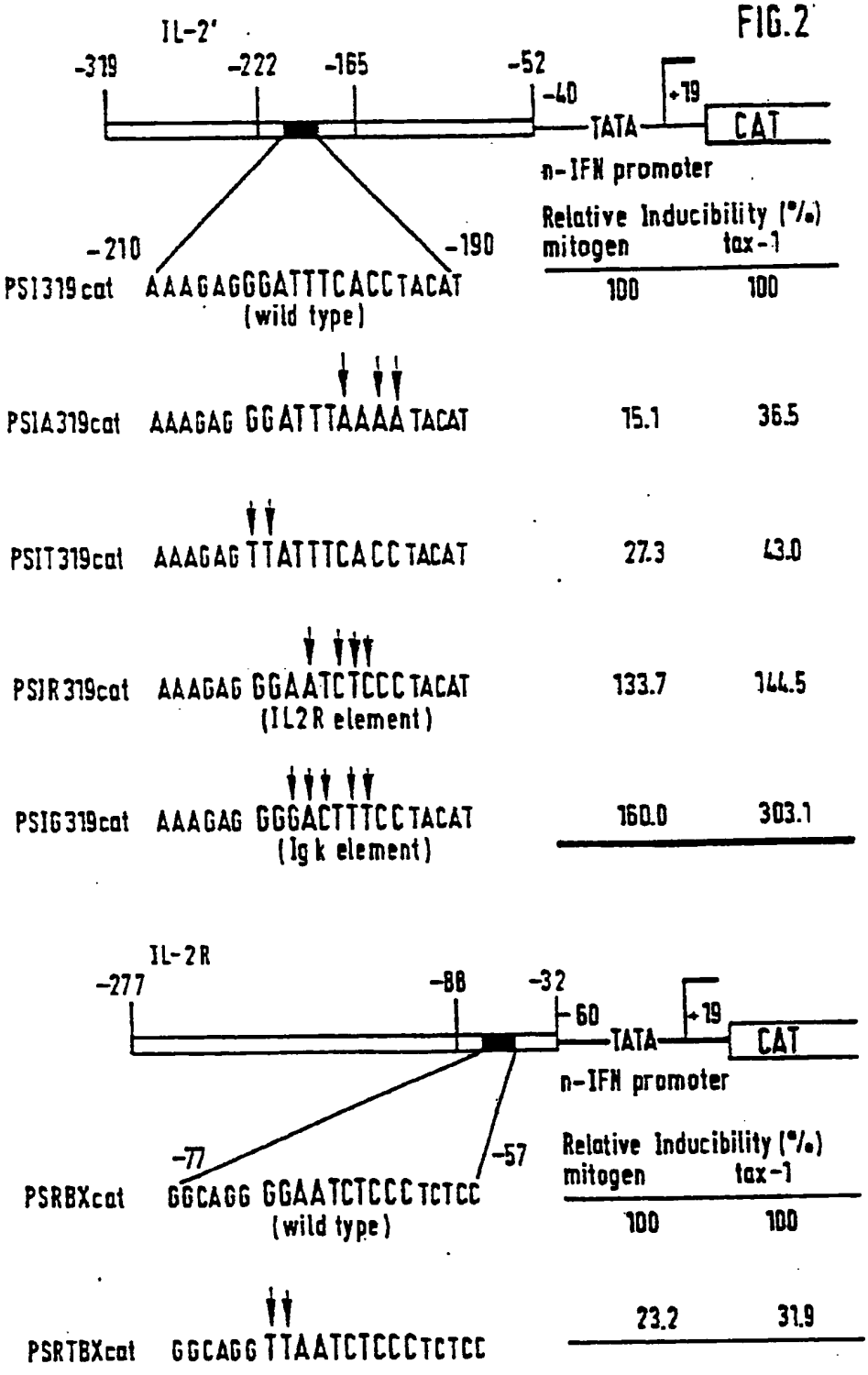


FIG.1b

FIG.2


IL-2'
-319
-222
-165
-52
-40
+19
TATA
CAT
n-IFN promoter
-210
-190
Relative Inducibility (%)
mitogen
tax-1
PS1319cat AAAGAGGGATTTCACCTACAT
(wild type)
100
100
PS1A319cat AAAGAG GGATTTAAAA TACAT
15.1
36.5
PSIT319cat AAAGAG TTATTTCACC TACAT
27.3
43.0
PSIR319cat AAAGAG GGAATCTCCC TACAT
(IL2R element)
133.7
144.5
PSIG319cat AAAGAG GGGACTTTCC TACAT
(Ig k element)
160.0
303.1
IL-2R
-277
-88
-32
-60
+19
TATA
CAT
n-IFN promoter
-77
-57
Relative Inducibility (%)
mitogen
tax-1
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31.9

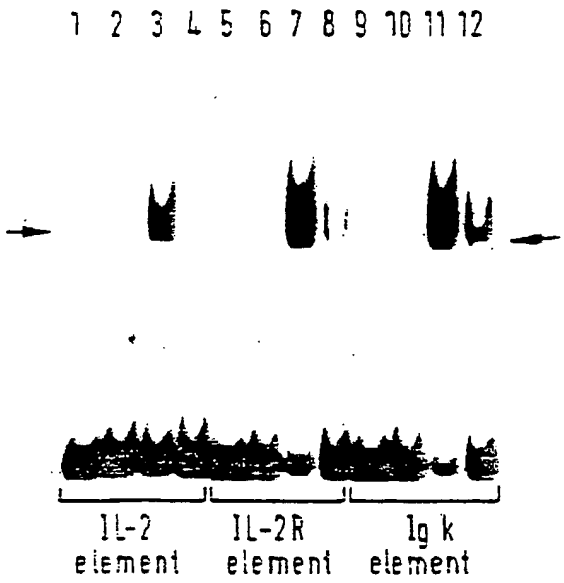

FIG. 3a

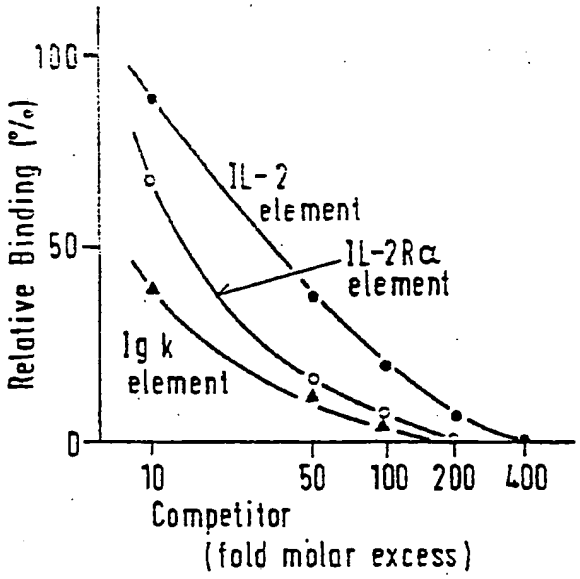

FIG. 3b

REGULATION OF EXPRESSION

FIELD OF THE INVENTION

The invention relates to the inhibition of expression of Interleukin-2 (IL-2) or Interleukin-2 α receptor (IL-2Rα) genes, or the inhibition of retrovirus replication. In particular, the invention is directed to a method for inhibiting HIV-1 and/or HTLV-1 using DNA or RNA fragments or compositions thereof which bind to transcription factors responsible for retroviral replication.

BACKGROUND OF THE INVENTION

Interleukin-2 (IL-2) plays an essential role in the clonal expansion of antigen-activated T-lymphocytes (T cells). In fact, the gene expression of both IL-2 and IL-2 α receptor (IL-2R α, p55, CD25) is transiently induced by antigen. Hence both the ligand and the receptor genes appear to be regulated in a coordinated manner to ensure a controlled clonal proliferation (Smith, K. A., *Ann. Rev. Immunol.* 2:319–334, (1984), Grene, W. C. & Leonard, W. J., *Ann. Rev. Immunol.* 4:69–95 (1986) and Taniguchi, T., *Ann. Rev. Immunol.* 6:439–464 (1980)).

The activation of IL-2 and the positive regulation of retroviral LTR-regions (HIV) have been described (Crabtree, G. R. et al., *Science, July* 1988). Four regulatory sequences were identified. Two regulation sequences for T-cell activation were found to span between −288 to −267 and −263 to −290 and were named "antigen receptor responsive elements" (ARRE 1 and 2). The factor that binds to ARRE 2 in Jurkat cells was named "nuclear factor of activated T cells" (NFAT). The formation of the factor was determined to be dependent upon protein biosynthesis. Studies have shown that NFAT-1 binds to the HIV-1-LTR region from −342 to −154. Various different factors are thought to bind to ARRE 1.

The nucleotide sequence of HIV-1 (Stareich, et al., *Science* 227:538–540 (1985)); HTLV-I (Seiki, et al., *Proc. Natl. Acad. Sci., USA* 80:3618–3622 (1983)); IL-2 (Fujita, et al., *Proc. Natl. Acad. Sci., USA* 80:7437–7441 (1983)); and IL-2R α chain (Cross, et al., *Cell*49:47–56 (1987)) have previously been described.

NOTE

The numbering of the nucleotides for the IL-2R α gene used in this application is based on the designation of the nucleotide corresponding to the most upstream cap site as +1 (see Hasagawa et al., "Structure and regulation of the genes encoding interleukin-2 and its receptor. In Regulation of Immune Gene Expression, M. Feldman and A. McMichael, eds (New York: The Humana Press), pp. 85–93 (1986) and Maruyama et al., Cell, Vol. 48, 343–350, Jan. 30, 1987, and Cross et al., Cell, 49, 47–56, 1987 at page 56 "Note added in Proof").

SUMMARY OF THE INVENTION

To elucidate the mechanism(s) of the coordinated gene expression for IL-2 and IL-2R α, the inventors have investigated for the presence of potential transcription factors that specifically interact with DAN regulatory elements. As a result of these experiments the inventors have surprisingly found three such regulator (transcription) factors as well as two regulatory sequences in the IL-2 gene and an upstream regulatory sequence (element) in the IL-2R α gene. The inventors have also unexpectedly discovered that recognition sites in HIV-1 and HTLV-1, c-fos and some of the lymphokines were homologous to one of the regulatory sequences of the IL-2 gene.

The present invention therefore relates to the inhibition or control of the IL-2 and IL-2R α genes and to the control or inhibition of fundamental cellular processes responsible for retrovirus replication, in particular HIV-1 and HTLV-1. The present invention is directed to nucleic acid or nucleic acid compositions including double stranded DNA, single stranded DNA or RNA which competitively bind to transcription factors. These transcription factors are required for the expression of IL-2 and IL-2R α as well as for retroviral replication. By competitively binding these transcription factors with DNA or RNA fragments or compositions thereof, the present invention makes it possible to regulate or inhibit the function of these factors by limiting their availability in vivo or in vitro.

The present invention thus relates to the inhibition or control of expression of IL-2 or IL-2R α genes, or the inhibition or control of retrovirus replication. In particular, the invention is directed to a method for inhibiting retroviral replication thereby providing a method of treating HIV-1 or HTLV-I infection in vivo.

The invention is specifically directed to: a DNA fragment of the IL-2 gene, spanning from −195 to −204 of the genetic sequence of said gene; a DNA fragment of the IL-2 gene, spanning from −115 to −164 of the genetic sequence of said gene; a DNA fragment of the IL-2 gene, spanning from −165 to −222 of the genetic sequence of said gene; a DNA fragment of the IL-2R α gene, spanning from −62 to −71 of the genetic sequence of said gene; and a DNA fragment of the IL-2R α gene, spanning from −32 to −86 of the genetic sequence of said gene.

The invention is also directed to a DNA molecule comprising two fragments of the IL-2 gene, said first fragment spanning from −195 to −204 of the genetic sequence of said gene, and said second fragment spanning from −115 to −164 of the genetic sequence of said gene but specifically lacking a substantial portion of the fragment spanning from −165 to −194 of the genetic sequence of said gene. These two fragments may further comprising a fragment of the IL-2R α gene, said fragment spanning from −62 to −71 of the genetic sequence of said gene.

The present invention further relates to: a DNA molecule comprising a fragment of the IL-2 gene and a fragment of the IL-2R α gene, said first fragment spanning from −195 to −204 of the genetic sequence of said IL-2 gene, and said second fragment spanning from −62 to −71 of the genetic sequence of said IL-2R α gene; a DNA molecule comprising a fragment of the IL-2 gene and a fragment of the IL-2R α gene, said first fragment spanning from −115 to −164 of the genetic sequence of said IL-2 gene, and said second fragment spanning from −62 to −71 of the genetic sequence of said IL-2R α gene; and a DNA molecule comprising a fragment of the IL-2 gene and a fragment of the IL-2R α gene, said first fragment spanning from 165 to −222 of the genetic sequence of said IL-2 gene, and said second fragment spanning from −32 to −86 of the genetic sequence of said IL-2R α gene.

Thus, the nucleotides of the present invention make it possible to competitively bind cellular transcription factors in vivo or in vitro. By competitively binding these transcription factors in vivo, this invention provides a method to inhibit retroviral replication in the

cell. Therefore, one important application of the present invention is to provide a novel treatment for patients suffering from HIV-1 or HTLV-I infection.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1*a* shows the gel-retardation assays for the detection of a transcription factor that specifically binds to both IL-2 and IL-2R α sequences.

FIG. 1*b* shows the results for methylation interference studies to determine the contact regions of transcription factor in the IL-2 and IL-2R α sequences.

FIG. 2 shows the results of in vivo expression studies using the IL-2 and IL-2R regulatory sequences and mutants thereof.

FIG. 3*a* shows the results of the gel-retardation analysis of the wild-type and mutant IL-2 DNA sequences with the nuclear extracts from the mitogen-stimulated Jurkat cells.

FIG. 3*b* shows the relative binding affinities of the transcription factor to the following DNA sequence elements: Igκ; IL-2R α; and IL-2.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention we provide an novel DNA molecule comprising: a) a fragment of the IL-2 gene, said fragment comprising the sequence spanning from −195 to −204 of said IL-2 gene, and/or the sequence spanning from −115 to −164 of said IL-2 gene: and/or b) a fragment of the IL-2R α gene, said fragment comprising the sequence spanning from −62 to −71 of the said IL-2R α gene.

The DNA molecule described in the previous paragraph may also include: a) a fragment of the IL-2 gene wherein said fragment includes the sequence spanning from −165 to −222 of said IL-2 gene; and/or b) a fragment of the IL-2R α gene wherein said fragment includes the sequence spanning from −32 to −86.

The DNA molecule may comprise two or more repeat sequences of said fragment or fragments of said IL-2 gene and/or of said IL-2R α gene or said derivatives and mutants thereof. Suitably up to 15, up to 10, or up to 4 of said repeat sequences may be present.

The DNA molecule of this invention may be single stranded (ss) or double stranded (dd). The invention further provides an RNA fragment which contains a sequence capable of hybridizing to at least one of the DNA molecules described in the present invention and which is also capable of hybridizing to the LTR of HIV-1.

Preferably the RNA molecule further contains an additional nucleotide sequence capable of hybridizing to a DNA sequence within the LTR region of HIV-1. This additional fragment hybridizes to sequences adjacent to but not overlapping with the LTR regions which hybridize with the DNA molecules described in the invention.

It is recognized that molecules or fragments of DNA or RNA as described in this invention may be altered by deletion, addition or mutation. A mutation or mutant as defined herein is any change or number of changes that alter the sequence of bases along the DNA or RNA fragment. Therefore, derivatives of the DNA or RNA sequence are encompassed by the present invention as long as the sequences can bind to regulatory (transcription) factor(s) to inhibit or control retroviral replication, or inhibit or control the expression of IL-2 or IL-2R α genes.

A fragment as used herein refers to a part or portion of the nucleotide sequence in and around the gene of interest. The boundaries of these nucleic acids (two or more nucleotides) have been defined according to the nucleotide position, for example, −195 to −204.

The boundaries as defined in this invention are not to be exclusive and can vary at the 5' or 3' end of the nucleotide sequence as long as these sequence fragments can bind to regulatory (transcription) factor(s) to inhibit or control retroviral replication, or inhibit or control the expression of IL-2 or IL-2R α genes. In addition, a double stranded DNA and/or RNA molecule as defined herein may also contain single stranded overhanging ends at the 3' termini, the 5' termini or both.

A further aspect of the invention comprises a composition for inhibiting the replication of HIV-1 or HTLV-I virus comprising a DNA molecule or RNA fragment as defined in this invention or a derivative or mutant thereof.

A further aspect of the present invention comprises a method of determining the binding affinity of a test substance. Test substances may consists of the nuclear (transcription) factor, analogous artificial protein factor, or RNA fragment as described in the invention. Binding affinity test may also be preformed on a peptidomimetic to regulatory sequences including an IL-2 regulatory sequence (−165 to −222; and/or −115 to −164), an IL-2R α gene sequence (−32 to −86), or to a recognition site sequence in HIV-1 and HTLV-I DNA. This method of determining binding affinities comprises bringing a DNA molecule as defined in the present invention into contact with said test substance and then determining the efficiency with which binding has occurred, if at all.

The foregoing DNA molecules may be obtained either by digestion of naturally occurring DNA from any cell containing the IL-2 or IL-2R α genes, for example, Jurkat cells. These DNA molecules may also be produced through cDNA synthesis by reverse transcription of mRNA extracted from suitable cells, or by chemical synthesis of single stranded DNA followed by formation of double stranded DNA using techniques which are now well known in the art. The RNA fragments can also be prepared by methods well known in the art. (Maniatis et al., Molecular Cloning, A Laboratory Manual.)

Other suitable DNA molecules for the purposes of the present invention comprise double stranded DNA mutants of the aforementioned DNA sequences wherein one or more of the nucleotides is substituted by an altered nucleotide and/or the factor (recognition) binding site of the aforementioned DNA sequences is multimerized. Such mutant DNA is capable of binding a nuclear (transcription) factor which binds to one or the other of the aforementioned IL-2 or IL-2R α gene sequences.

One such mutant double stranded DNA has the sequence:

5' CTAGAGGGATTTCACCGAGGGATTTCACCGAGGGATTTCACCGAGGGATTTCACCG 3'
TCCCTAAAGTGGCTCCCTAAAGTGGCTCCCTAAAGTGGGTCCCTAAAGTGGCCTAG 56MER
x2

factor binding site (_____)

Thus it is possible to create, artificially, DNA sequences that efficiently bind to the transcription factors that are involved not only in binding recognition sequences of the IL-2 and IL-2R α genes but also involved in binding recognition sequences in HIV-1 and HTLV-I. These transcription factor(s) may also bind other genes in T lymphocytes as well as other cells. Thus, by competitively binding these factors, it is possible to block their ability to bind to IL-2 and IL-2R α genes as well as prevent their binding to recognition sequences in HIV-1 or HTLV-I. The result described here is merely given by way of example and it is possible to create DNA molecules which are even more efficient in binding these transcription factors.

The present invention also relates to RNA fragments capable of binding to the LTR of HIV-1 sequence. Such an RNA fragment may contain any number of nucleotides. for example, 30 nucleotides and may be blocked at the 5' end. The resulting RNA/DNA hybrid binds the regulatory (transcription) factor with reduced affinity or binding is blocked altogether. By designing the RNA molecule so that it is specific for the sequences adjacent to the recognition site of the LTR, binding to the recognition sequences of IL-2 or IL-2R α genes or other genes can be avoided or will occur with reduced affinity. This is possible because the sequences surrounding the recognition sites of IL-2 and IL-2R α differ from those surrounding the recognition sites in the LTR of HIV-1.

Specific nucleic acid fragments or molecules such as ssRNA, ssDNA and dsDNA molecules can be made with altered solubility characteristics, such as increased lipophilicity for easier penetration through biological membranes. Improved lipophilicity can be achieved by modifying the ribose or deoxyribose residues with lipophilic compounds, for example. terpene. The competitive nucleic acid fragments or molecules described in the present invention may also be obtained by using rare nucleotides or nucleotides analogues known in the art. Such artificial DNA or RNA sequence or multimeric forms thereof may be used for competitive titration of the regulator (transcription) factor(s) in the cell.

The nucleic acid molecules or fragments of the present invention open up the possibility of therapy of HIV-1 or HTLV-I infections by administration of DNA which will bind competitively to the factor(s) which induce viral replication such as tax-1. Therapy for HIV-1 or HTLV-I infection may also be accomplished by administering an RNA or artificial protein or fragment thereof, for example, a peptide or peptidomimetic which will bind to the LTR of HIV-1. The invention also makes it possible to carry out binding studies on synthetic binding proteins which will bind competitively to the said sequences of the cellular DNA without having inducing activity, thus in this way down regulating virus production.

The subject nucleic acids can be formulated into pharmaceutical compositions according to known methods of preparing pharmaceutically useful compositions. In this manner, the nucleic acids are combined in a mixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, including other human proteins, e.g., human serum albumin, are described, for example, in *Remington's Pharmaceutical Sciences* (16th ed., Osol, A., ed., Mack, Easton, Pa. (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain a therapeutically effective amount of the subject nucleic acids (an effective amount for controlling or inhibiting expression of IL-2 and IL-2R α genes as well as inhibiting or controlling HIV-1 or HTLV-I viral replication), together with a suitable amount of carrier vehicle.

The nucleic acids may be formulated as a sterile pharmaceutical composition for therapeutic use which is suitable for intravenous administration. The product may be in lyophilized form to be reconstituted for use by the addition of a suitable carrier, or diluent, or alternatively, it may be in the form of an aqueous solution.

For reconstitution of a lyophilized product in accordance with the present invention, one may employ a sterile diluent, which may contain materials generally recognized for approximating physiological conditions. In this manner, the sterile diluent may contain a buffering agent to contain a physiologically acceptable pH, such as sodium chloride, saline, phosphate-buffered saline, and/or other substances which are physiologically acceptable and/or safe for use.

When used as an aqueous solution, the pharmaceutical composition, for the most part, will contain many of the same substances described above for the reconstitution of a lyophilized product.

The nucleic acids useful in the methods of the present invention may be employed in such forms as, for example sterile suspensions for injection or encapsulated for targeting to specific tissue sites. The nucleotides may also be conjugated with antibodies directed to cell surface structures of T-cells or other cells which maybe infected with HIV-1 or HTLV-I. See, for example, Bevilacqua et al., *PNAS USA* 83:9238–9242 (1987); Cotran et al., *J. Exp. Med.* 164:661–666 (1986).

Where the subject nucleic acids are to be administered to a host for controlling or inhibiting expression of IL-2 and IL-2R α as well as inhibiting or controlling HIV-1 and HTLV-I viral replication, the nucleic acids may be administered, for example, intraarticularly, intraperitoneally, intrapleurally, intraocularly, by injection, subcutaneously, or the like. Administration by injection includes continuous infusion as well as single or multiple boluses.

The amount of the subject nucleic acid administered will vary with the manner of administration, the concurrent use of other active compounds, host size, type and severity of infection, and the like. Generally, the nucleic acids will be administered in sufficient doses to obtain a concentration of about 0.1 nM to about 100 nM, usually about 5 nM of the nucleotide in the blood. The dosage amount of nucleic acids necessary to obtain the desired concentration in the blood can be determined by pharmacokinetic studies using labeled nucleotides. Alternatively, in vivo studies on test animals, for example, monkeys may be used to determine effective dose ranges.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved through the use of polymers to complex or absorb the subject nucleic acids. The controlled delivery may be achieved by selecting appropriate macromolecules (for example, polyesters, polyamino acids, polyvinyl, polypyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate). The appropriate concentration of macromolecules as well as the methods of incorporation may be determined using the above-mentioned pharmacokinetic or in vivo studies. In this manner release of the nucleic acids can be controlled.

Another possible method useful in controlling the duration of action by controlled release preparations is the incorporation of the subject nucleic acids into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid), or ethylenevinylacetate copolymers.

Alternatively, instead of incorporating the subject nucleotides into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatine-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* (1980).

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

Mitogen-induced Binding of Nuclear Factor to the Regulatory Sequence Elements of Human IL-2 and IL-2R α Gene

Nuclear extracts were prepared according to the method of Dignam et al. (Dignam, J. D. et al., *Nucleic Acids Res.* 11:1475–1489 (1983)), and the tel-retardation assay performed as previously described (Signh, H. et al., *Nature* 319:154–158 (1986); Fujita, T. et al., *EMBO J.* 7, in press (1988)). The ^{32}P-labelled IL-2 DNA probe (phosphorylated at the 5'terminus of the coding strand) was prepared by isolating a 58 bp XbaI-DraI fragment from pIL2-222cat (Fujita, T. et al., *Cell* 46:401–407 (1986)). It encompasses the region from −165 to −222 with respect to the CAP site. The IL-2R α DNA probe was similarly prepared by isolating a 56 bp SalI-BamHI fragment from pIL-2R86cat. Essentially, the IL-2(R α probe encompasses the region from −32 to −86 with respect to the most upstream CAP site (Maruyama, M. et al., *Cell* 48:343–350 (1987)). Specific activity of the DNA was 3,000 c.p.m./f.mole in both probes. The unlabelled competitor DNAs were prepared similarly. Methylation interference analysis was carried out as described previously (Fujita, T. et al., *EMBO J.* 7, in press (1988)). The DNA probes were exactly the same as those described above, except that they were labelled at the 5' terminus of the coding or non-coding strand.

The results are presented in FIG. 1, wherein FIG. 1*a* shows gel-retardation assays for the detection of a factor that specifically binds to both IL-2 and IL-2R α sequences. Nuclear extracts (10 μg) from unstimulated or mitogen-stimulated Jurkat cells (1 μg/ml PHA, 50 ng/ml TPA for 3 hrs) were incubated with either IL-2 or IL-2R α DNA probe alone (3 f.moles) or in the presence of unlabelled competitor DNAs. Lanes 1 to 5 contain the IL-2 DNA probe, while lanes 6 to 10 contain the IL-2R α probe. Lanes 1 and 6, no extract; Lanes 2 and 7, extract from uninduced cells (−); Lanes 3 and 8, extract from induced cells (+); Lanes 4 and 9, extract (+) and unlabelled IL-2 DNA (1.2 p.mole); Lanes 5 and 10, extract (+) and unlabelled IL-2R α DNA (1.2 p.mole); FIG. 1*b* shows the results for methylation interference analysis for the factor contact regions of the IL-2 and IL-2R α DNAs. The left panel shows the analysis of the coding and noncoding strand of the IL-2 probe and the right panel, the analysis of the IL-2R α probe. The positions of methylated guanine residues that interfered with the factor binding are marked.

Using the gel-retardation assay at least three nuclear (transcription) factors were detected in a human T cell line, Jurkat that specifically bind to regulator elements within the 5'flanking region of the human IL-2 gene. The three factors recognize distinct DNA sequences. The DNA binding activities of two such factors were found to be induced in the mitogen-stimulated cells. The specific binding of one of the factors to the IL-2 gene sequence spanning from −165 to −222 was also inhibited by a molar excess of a DNA segment of the human IL-2R α regulatory sequences spanning from −32 to −86. Reciprocally, the factor binding to the IL-2R α DNA was inhibited by the IL-2 DNA segment (FIG. 1*a*). Other inducible factor(s) bound to the IL-2 sequence spanning from −115 to −164 and this binding was not affected by any of the tested IL-2R α sequences. The consensus sequences recognized by the factor were not obvious; examination of the factor contact sites of both DNA segments by methylation interference assay revealed as shown in FIG. 1*b*, that the contact regions of the factor were −195 to −204 and −62 to −71 for the IL-2 and IL-2R α genes, respectively. The contact region of the IL-2R α gene shows remarkable sequence similarity to the binding sites of NF- B that binds to an Igκ gene enhancer element (Sen, R. et al., *Cell* 46:705–716 (1986)). The corresponding region of the IL-2R α gene and a sequence within the HIV LTR also bind a transcription factor (NF- B-like factor or HIVEN86A) (Nabel, G. et al., *Nature* 326 711–713 (1987); Böhnlein, E. et al., *Cell* 53:827–836 (1988); Leung, K. et al., *Nature* 333:776–778 (1988)). Unexpectedly, we have found that the same factor binds to the 10 bp sites of this regulator sequence of IL-2.

EXAMPLE 2

To examine extent to which the factor binding to the above sequence elements influences the induced gene expression and the effect of various mutations within the factor contact region of the IL-2 and IL-2R α genes, various mutations within the elements were introduced and the functional properties of the mutant genes were analyzed using a strategy as follows:

Vector constructions were carried out essentially as described previously (Maruyama, M. et al., *Cell* 48:343–350 (1987)). To generate the mutations, the IL-2 or IL-2R α sequences were once cloned into a M13 phage vector and subjected to the oligonucleotide-directed mutagenesis as described by Kunkel et al., (Kunkel, T. A. et al., *Methods in Enzymology* 154:367–382 (1987)). Nucleotide sequences of the mutant DNAs were confirmed by sequence analyses. The reference gene, pRSVTK essentially contains the RSV LTR sequence linked to the HSV tk gene in the

pRSVcat backbone DNA (Gorman, C. et al., *Proc. Natl. Acad. Sci. USA* 79:6777–6781 (1982)). The tax-1 expression vector, pCDS contains the HTLV-I-pX coding sequence that is abutted downstream to the CMV promoter/enhancer sequences in the vector H3M (Aruffo, A. et al., *Proc. Natl. Acad. Sci. USA* 84:8573–8577 (1987)). The DNA transfection, mitogen stimulation, RNA preparation and S1 analysis were carried out essentially as described previously (Maruyama, M. et al., *Cell* 58:343–350 (1987)). In determining the relative inducibilities by the S1 analysis of mRNAs, the induced mRNA levels were normalized by the tk specific mRNA levels in each sample (the tk mRNA levels did not differ significantly from one sample to the other).

Referring to FIG. 2 the IL-2 and IL-2R α regulatory sequences were respectively excised out of pI319Bcat and pRBXBcat (Maruyama, M. et al., *Cell* 58:343–350 (1987)) and they were each introduced into pSVOcat (Gorman, C. et al., *Mol. Cell. Biol.* 2:1044–1051 (1982)) as depicted in FIG. 2. Mutant genes were similarly constructed as described below. The genes were each cotransfected into Jurkat cells with a reference gene, pRSVtk. The cells were then treated by mitogens. Alternatively, the vector DNAs were transfected with a tax-1 expression vector pCDS and the cells were not treated by mitogen. The gene expression level was monitored by S1 analysis of the induced mRNA. Assuming that 1% of the transfected cells had received DNAs, the induced mRNA levels were about 250 and 100 strands per cell in the mitogen-stimulated cells and the tax-1-expressing cells, respectively in the case of the IL-2 gene (pSI319cat). In the case of the IL-2R α gene (pSRBXcat) the values were respectively about 150 and 100 strands per cell. The values of pSRBXcat are significantly lower than those of pRPXBcat, due to the absence of additional upstream elements (Maruyama, M. et al., *Cell* 48:343–350 (1987)). The transfection experiments were repeated three times and the results were reproducible. Always in the absence of mitogen stimulation or tax-1 expression, no specific mRNA was detectable in the transfected cells.

In this series of mutants, we thus included mutants in which the 10 bp IL-2 gene element was replace with the corresponding 10 bp element of either the IL-2R α or the Igκ gene ("sequence swapping" experiments). In this experiment, we also examined the effect of HTLV-I-derived transactivator, tax-1 on the expression of those genes. The tax-1 ($p40^x$, tax-1) has been shown to activate the IL-2 and IL-2Rα genes without extracellular mitogenic stimulation of T cells (Maruyama, M. et al., *Cell* 48:343–350 (1987); Inoue, J. et al., *EMBO J.* 5:2883–2888 (1986); Cross, S. L. et al., *Cell* 49:47–56 (1987); Siekevitz, M. et al., *Proc. Natl. Acad. Sci. USA* 84:5389–5393 (1987)). A significant reduction of the mitogen and tax-1 induced gene expression was observed with the IL-2 and IL-2R α genes each carrying mutations within the factor contact region. On the other hand, the IL-2 mutant genes each possessing the swapped sequence element gave rise to higher induction levels compared to the wild-type gene.

EXAMPLE 3

The upregulation of the IL-2 mutant genes was investigated by examining the binding affinities of the factor to the wild-type and mutant genes. A series of gel-retardation assays were preformed by using the factor extract and the IL-2 gene segments each containing either the wild-type or the swapped elements and the results are presented in FIG. 3, which shows that the Jurkat-derived factor binds the IL-2, IL-2R α and Igκ elements with different affinities.

In particular FIG. 3*a* shows the results of gel-retardation analysis of the wild-type and mutant IL-2 DNA segments with the nuclear extracts from the mitogen-stimulated Jurkat cells. Fifty-two bp DNA segments of either wild-type (lanes 1–4) (spanning from −222 to −173) or the similar DNAs containing the swapped IL-2R α (lanes 5–8) or Igκ (lanes 9–12) elements were chemically synthesized and subjected to the analysis exactly as described in FIG. 1. Lanes 1, 5, 9; probe DNA. Lanes 2, 6, 10; probe DNA and extract from unstimulated cells. Lanes 3, 7, 11; probe DNA and extract from mitogen-stimulated cells. Lanes 4, 8, 12; same as lanes 3, 7, 11; except that each sample received 400 fold molar excess of the unlabelled wild-type IL-2 DNA segment.

FIG. 3 shows inhibition of complex formation between the factor and wild-type IL-2 DNA by the DNAs each containing the factor recognition element of either IL-2 or IL-2R α or Igκ gene. Gel-retardation assays were performed with the labelled wild type IL-2 DNA segment as described above. The DNAs were exactly the same as described above. The efficacy of formation of factor-DNA complexes were quantitated by densitometric analysis of the autoradiogram. Complex formation in the absence of competitor DNAs was taken as 100%.

More particularly in FIG. 3*a* it is shown that the gel mobilities of the shifted bands were indistinguishable from each other, evidencing that the same factor is bound to each of the elements. Moreover, under identical assay conditions, the intensity of the bands was significantly different and followed in increasing order Igκ, IL-2R α (IL-2 respectively. Next, a DNA competition assay was carried out in which the degree of complex formation between the factor and the ^{32}p-labelled, wild-type IL-2 gene segment was analyzed in the presence or absence of unlabelled IL-2 gene segments. As shown in FIG. 3*b*, the affinities of the factor to those DNA sequences are in the order of Igκ, IL-2R α and IL-2 elements. Thus, the observed upregulation of the mutant genes may be attributed to a higher binding affinity of the factor to the Igκ and IL-2R α elements. As expected, the factor showed greatly reduced affinities to the genes with down-mutations factor. Significantly, the sequence elements of the human and murine IL-2 genes are identical, suggesting the importance of strict conservation of the element in controlling gene expression. These findings show that the gene expression level is a function of the binding affinity of the fragment to the regulatory element(s).

The induced expression of the IL-2 and IL-2R α genes thus involves a common transcription factor whose binding activity to both genes increases in mitogen-stimulated Jurkat T cells. We have noticed that the binding activity becomes almost undetectable following cycloheximide treatment at the onset of mitogen stimulation, suggesting the requirement of the de novo synthesis of this factor.

Interestingly, similar sequence elements are noticeable within the promoter region of many lymphokine genes as well as the LTR regions of lymphotropic retroviruses (Table 1). In view of the considerable sequence divergence in the contact sites of the factor as described above, this factor is acting also on the above

listed genes as a "universal" regulatory factor. In this context, it is worth noting that many if not all of the listed genes are also activated by tax-1 (Maruyama, M. et al., *Cell* 48:343–350 (1987); Inoue, J. et al., *EMBO J.* 5:2883–2888 (1986); Cross, S. L. et al., *Cell* 49:47–56 (1987); Siekevitz, M. et al., *Proc. Natl. Acad. Sci. USA* 84:5389–5393 (1987); Yoshida, M. et al., *Ann. Rev. Immunol.* 5:541–559 (1987); Siekevitz, M. et al., *Science* 238:1575–1578 (1987); Miyatake, S. et al., *Nucleic Acids Res.* 16:6547–6566 (1988)).

EXAMPLE 4

In order to examine whether the IL-2 regulatory sequence that is bound by the transcription factor is useful in (i) detection of the factors that bind to the similar sites in other genes, and (ii) titration of the factor by DNA sequences which efficiently bind to the factor (useful in the selective inhibition of certain gene expression), a piece of double stranded DNA was chemically synthesized as depicted below. The DNA consists of two strands each consisting of 56 nucleotides. The double stranded DNA contains four repeats of the factor binding site. It is possible to create a variety of similar DNA, in order to increase the binding efficiency to the factor.

5' CTAGAGGGATTTCACCGAGGGATTTCACCGAGGGATTTCACCGAGGGATTTCACCG 3'
TCCCTAAAGTGGCTCCCTAAAGTGGCTCCCTAAAGTGGCTCCCTAAAGTGGCCTAG 56mer
×2

factor binding site (_____)

(1) When this DNA was used as a competitor DNA in a gel shift analysis as described in FIG. 1*a*, the appearance of the shifted band, which reflects the complex formed between the factor and the IL-2 DNA (labelled by ^{32}P), was completely inhibited by the unlabelled, molar excess of the above DNA. This demonstrates that the above DNA binds to the factor.

(2) When the above DNA was ^{32}P labelled and used as the probe to perform the gel shift assay under exactly the same assay conditions as described in FIG. 1*a*, the specific complex with the DNA and the factor was also obtained. This complex formation was inhibited by the molar excess of the IL-2 and IL-2 α receptor DNA sequences containing the factor binding site. The results corroborate the result presented in (i), that the above DNA can bind to the factor. Furthermore, in the gel shift analysis, an additional band is seen in the gel shift assay, indicating that the DNA can bind more than two of the factors (since the above DNA contains multiple factor binding sites).

(3) When the above DNA was linked to the IFN promoter, exactly as described with reference to FIG. 2, instead of the natural IL-2 and IL-2 α receptor DNA sequences, and assayed for the inducibility of the reporter CAT gene by transfecting the constructed DNA into Jurkat cells (the cells were subsequently induced by mitogens as described above), very efficient induction of the CAT gene was observed. In fact, the inducibility was about 15 times higher compared to the similar construct containing the natural IL-2 sequence (in FIG. 2, it is pSIR319cat).

Thus it is possible to create, artificially, DNA and other sequences that efficiently bind the transcription factor which is involved in the recognition of IL-2 and IL-2 α receptor genes, and of other genes in T lymphocytes and other cells as well as recognition sites in the HIV-1 and HTLV-I. The results described here are given by way of example and it is possible to create even more efficient DNA that bind to the factor, thereby blocking the factors binding to IL-2 and IL-2 α receptor genes and the other aforementioned recognition sequences.

I claim:

TABLE 1

	Presence of factor recognition sites within promoter, LTR regions of the genes expressed in T cells.		ref.
IL-2	(human)*	$-^{204}$GGATTTCACC$-^{195}$	Fujita, T. et al. Cell 46, 401–407 (1986).
IL-2R α	(human)*	$-^{71}$GGAATCTCCC$-^{62}$	Maruyama, M. et al. Cell 48, 343–350 (1987).
IFN-γ	(human)	$-^{233}$GAATCCCACC$-^{224}$	Gray, P. W. & Goeddel, D. V. Nature 298, 859–863 (1982).
IL-6	(human)	$-^{134}$GGATTTTCCC$-^{125}$	Yasukawa, K. et al. EMBO J. 6, 2939–2945 (1987).
IL-3	(mouse)	$-^{295}$GAGATTCCAC$-^{286}$	Miyatake, S. et al. Proc. Natl. Acad. Sci. USA 82, 316–320 (1985).
IL-4	(mouse)	$-^{190}$GGTGTTTCAT$-^{181}$	Otsuka, T. et al. Nucleic Acids Res. 15, 334–344 (1987).
GM-CSF	(mouse)	$-^{104}$GAGATTCCAC$-^{95}$	Stanley, E. et al. EMBO J. 4, 2569–2573 (1985).
c-fos	(human)	$-^{278}$GGCCTTTCCC$-^{269}$	Van Straaten, F. et al. Proc.

TABLE 1-continued

	Presence of factor recognition sites within promoter, LTR regions of the genes expressed in T cells.	ref.
HIV LTR*	$-^{89}$GGGACTTTCC$-^{80}$ $-^{103}$GGGACTTTCC$-^{94}$	Natl. Acad. Sci. USA 80, 3183-3187 (1983). Starchch, H. et al. Science 227, 538-540 (1985).
HTLV-I LTR	$-^{168}$GGAGCCTACC$-^{177}$ $-^{244}$GACGTCTCCC$-^{235}$	Seiki, M. et al. Proc. Natl. Sci. USA 80, 3618-3622 (1983).

*Factor binding has been demonstrated.

1. A DNA molecule comprising a fragment of the IL-2 gene, said fragment spanning from −195 to −204 of the genetic sequence of said gene and mutants thereof.

2. A DNA molecule comprising a fragment of the IL-2 gene, said fragment spanning from −115 to −164 of the genetic sequence of said gene and mutants thereof.

3. A DNA molecule comprising two fragments of the receptor (IL-2R) α gene, said fragment spanning from −62 to −71 of the genetic sequence of said gene and mutants thereof.

4. A DNA molecule comprising a fragment of the IL-2 gene, said first fragment spanning from −195 to −204 of the genetic sequence of said gene, and said second fragment spanning from −115 to −164 of the genetic sequence of said gene and mutants thereof but specifically lacking a substantial portion of the fragment spanning from −165 to −194 of the genetic sequence of said gene.

5. A DNA molecule comprising a fragment of the IL-2 gene and a fragment of the IL-2R α gene, said first fragment spanning from −195 to −204 of the genetic sequence of said IL-2 gene, and said second fragment spanning from −62 to −71 of the genetic sequence of said IL-2R α gene and mutants thereof.

6. A DNA molecule comprising a fragment of the IL-2 gene and a fragment of the IL-2R α gene, said first fragment spanning from −115 to −164 of the genetic sequence of said IL-2 gene, and said second fragment spanning from −62 to −71 of the genetic sequence of said IL-2R α gene and mutants thereof.

7. The DNA molecule of claim 4 further comprising a fragment of the IL-2R α gene, said fragment spanning from −62 to −71 of the genetic sequence of said gene and mutants thereof.

8. A DNA molecule comprising a fragment of the IL-2 gene, said fragment spanning from −165 to −222 of the genetic sequence of said gene and mutants thereof.

9. A DNA molecule comprising a fragment of the receptor (IL-2R) α gene, said fragment spanning from −32 to −86 of the genetic sequence of said gene and mutants thereof.

10. A DNA molecule comprising a fragment of the IL-2 gene and a fragment of the IL-2R α gene, said first fragment spanning from −165 to −222 of the genetic sequence of said IL-2 gene, and said second fragment spanning from −32 to −86 of the genetic sequence of said IL-2R α gene and mutants thereof.

11. A DNA molecule as defined in claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 which is a double stranded DNA molecule.

12. A DNA molecule as defined in claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, comprising two or more of at least one of said fragments.

13. A DNA molecule as defined in claim 12 which is a double stranded DNA molecule.

14. A DNA molecule as defined in claim 12, containing up to 15 of at least one of said fragments.

15. A DNA molecule as defined in claim 14 which is a double stranded DNA molecule.

16. A DNA molecule as defined in claim 14, containing up to 10 of at least one of said fragments.

17. A DNA molecule as defined in claim 16 which is a double stranded DNA molecule.

18. A DNA molecule as defined in claim 16, containing up to 4 of at least one of said fragments.

19. A DNA molecule as defined in claim 18 which is a double stranded DNA molecule.

20. A RNA fragment comprising a sequence capable of stably hybridizing at least one of the DNA sequences as defined in claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

21. An RNA fragment defined in claim 20 which is capable of stably hybridizing to the LTR region of HIV-1.

22. An RNA fragment comprising a sequence capable of stably hybridizing to at least one of the DNA sequences as defined in claim 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, said RNA fragment also being capable of stably hybridizing to the LTR region of HIV-1, said RNA fragment which further contains an additional nucleotide sequence, said sequence being capable of hybridizing to a region adjacent to the LTR region of HIV-1.

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